

Leonie Mekenkamp

**Studies in colorectal cancer
metastases: implications for
clinical practice**

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Colofon

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Studies in colorectal cancer metastases: implications for clinical practice

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Chapter 1 **General
introduction and outline
of the thesis**

INTRODUCTION

Colorectal cancer (CRC) is the second most common cause of cancer death in the Western World, and the life time risk of developing sporadic CRC is 5-6%.¹ Approximately half of the CRC patients develop metastatic disease, either at diagnosis or during follow-up.² For patients with unresectable metastatic CRC there are no curative options, but significant benefit in survival can be achieved with systemic treatment. In recent years, the systemic treatment options for patients with metastatic CRC have changed considerably. For many decades 5-fluorouracil plus leucovorin (5-FU/LV) was the only available treatment regimen with median overall survival (OS) times of approximately 12 months.³ Oral fluoropyrimidines (capecitabine, uracil/tegafur) have shown to be as effective and better tolerated in comparison to intravenous 5-FU/LV.^{4,5} Therefore, capecitabine is a good alternative for 5-FU/LV, both when given as monotherapy and in combination schedules. The availability of oxaliplatin and irinotecan have further improved the median OS to 14-19 months. Prospective randomized clinical trials have shown that there is no preference for combined administration of these cytotoxic agents instead of their sequential use, at least in patients that are not eligible for secondary resections of metastases after downsizing and/or patients with good performance status allowing multiple lines of treatment.^{6,7} It is probably more important that patients are exposed to all three cytotoxic drugs (fluoropyrimidines, oxaliplatin, irinotecan) during the course of their disease, than that patients receive these drugs concurrently in first-line which also implies that (some of) these drugs have to be administered below their maximal tolerated dose. More recently, targeted agents have been approved for clinical use in metastatic CRC and these have added further benefit in OS. Bevacizumab, a humanized monoclonal antibody

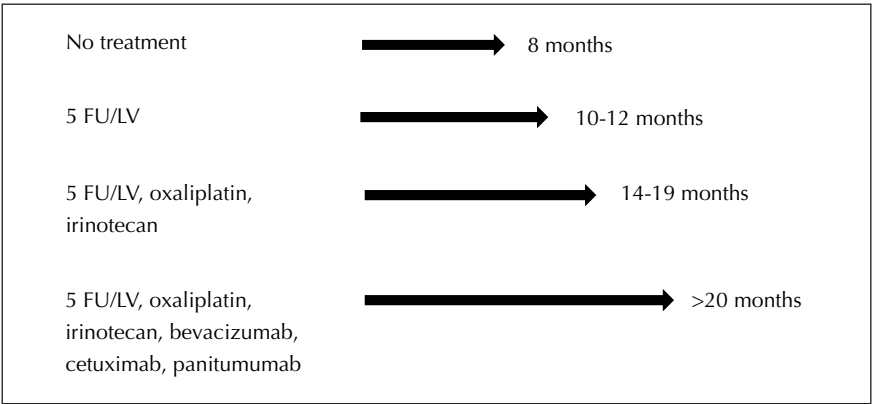


Figure 1. Median OS in metastatic CRC patients with the current available chemotherapy and targeted agents.

against vascular endothelial growth factor (VEGF), combined with 5-FU based chemotherapy is nowadays the standard first-line treatment for metastatic CRC.⁸⁻¹¹ In patients with *KRAS* wild type tumors, two antibodies against the epidermal growth factor receptor (EGFR), cetuximab and panitumumab, have shown their greatest absolute benefits as monotherapy in chemo-refractory patients^{12,13} and have also shown survival benefits in first-line or second-line treatment in combination with chemotherapy.¹⁴⁻¹⁷ The integration of targeted drugs in the standard treatment of patients with metastatic CRC has increased the median OS to approximately 2 years (Figure 1).

PROGNOSTIC FACTORS IN METASTATIC COLORECTAL CANCER

Clinical trials in metastatic CRC, although using similar patient selection criteria, often display a surprising heterogeneity in survival rates. This heterogeneity is usually explained by differences in patient and tumor-related factors, irrespective of treatment. Identification of prognostic factors in metastatic CRC is important because the estimation of individual prognosis based on patient and tumor characteristics may allow a more personalized treatment approach. In addition, appropriate stratification based on established prognostic factors will allow a more reliable interpretation of study results by prevention of heterogeneity in baseline patient characteristics.

Clinical prognostic factors

Patient characteristics influence clinical outcome in metastatic CRC. A variety of clinical parameters such as performance status^{18,19}, serum lactate dehydrogenase (LDH)^{6,20}, the number of metastatic sites involved^{21,22} and localization of the primary tumor^{23,24} have been identified as prognostic factors. The prognostic value of other factors, such as the onset of metastases (synchronous vs. metachronous), carcinoembryonic antigen (CEA), age, gender, and obesity, is less clear. There is no real consensus and general acceptance of prognostic factors, which is probably due to a high level of heterogeneity in clinical trials reporting prognostic factors. However, risk group assessment based on baseline clinical parameters in a large patient cohort has been performed, identifying performance status, white blood cell count, alkaline phosphatase, and number of metastatic sites as the most important prognostic factors.²⁴ Surprisingly, white blood cell count and serum alkaline phosphatase levels are not commonly used as stratification parameters in clinical trials. Therefore, re-establishing clinical prognostic factors in metastatic CRC is necessary and could reduce the heterogeneity in survival rates in

randomized clinical trials. The development of a clinical nomogram in which these prognostic factors are included would greatly assist individual patient management.

Histopathological prognostic factors

Tumor extent (T stage), lymph node status (N stage), histological subtype, differentiation grade, and the assessment of lymphatic and venous invasion are still the most important morphological prognostic factors in CRC²⁵, even in case of metastatic disease. Of these morphological features, lymph node status is the strongest prognostic factor and the main indicator for the application of adjuvant therapy. Throughout the years several guidelines have been issued suggesting a minimum number of lymph nodes to be evaluated to confirm node-negativity.^{26, 27} In CRC patients the retrieval of lymph nodes depends on biological characteristics, choice of treatment, and pathologic assessment of the specimen. Due to the prognostic value of lymph

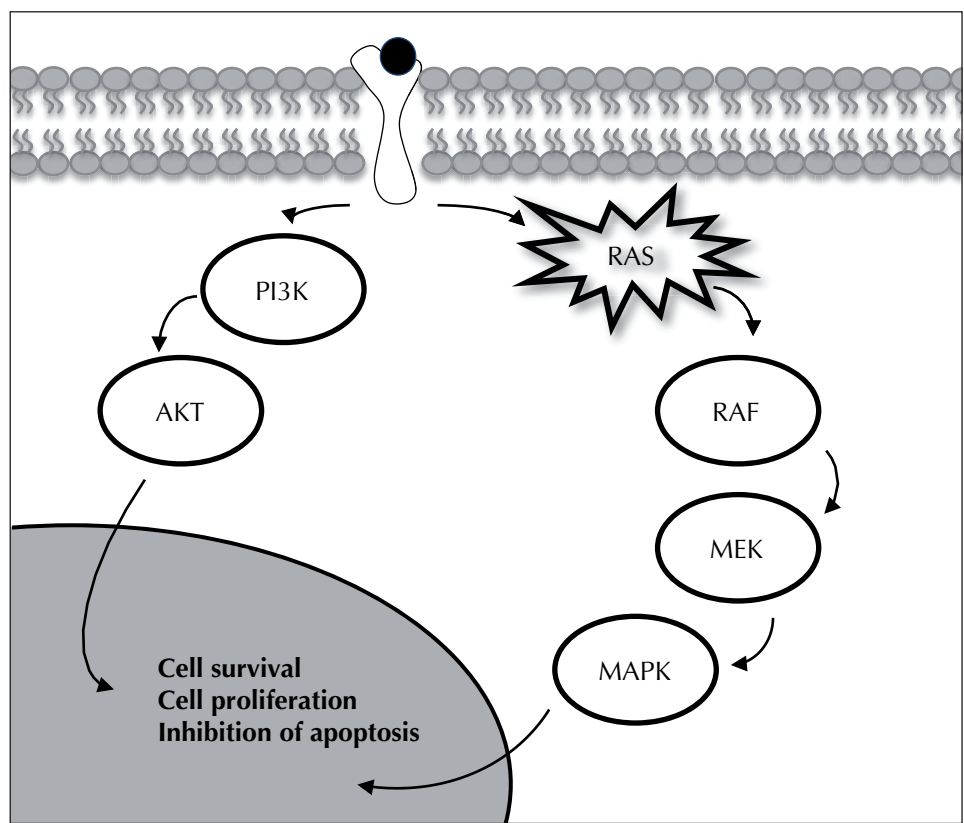


Figure 2. The RAS/RAF/MAPK signaling pathway resulting in the stimulation of cell proliferation and inhibition of apoptosis.

node retrieval, it is clinically relevant to optimize lymph node assessment in CRC patients. This issue is more problematic in patients with rectal cancer who have received neoadjuvant treatment, due to the treatment effects on lymph node status.

PREDICTIVE FACTORS IN METASTATIC COLORECTAL CANCER

Predictive markers indicate the likelihood of benefit from treatment, and are important to optimize the selection of metastatic CRC patients eligible for specific treatment regimes. Because treatment with chemotherapy and targeted agents is associated with potential (and sometimes severe) toxicity and high costs, it is important to establish markers that are predictive for efficacy. In this respect, the identification of *KRAS* mutation as a negative predictive marker for the response to anti-EGFR therapy has been one of the most recent important achievements in metastatic CRC. An oncogenic mutation in *KRAS* leads to constitutive activation of the RAS/RAF signalling pathway independent from EGFR activation by binding of the ligand²⁸, ultimately resulting in the stimulation of cell proliferation and the inhibition of apoptosis (Figure 2).²⁹ Metastatic CRC patients with tumors harboring a *KRAS* mutation are resistant to treatment with anti-EGFR antibodies (panitumumab and cetuximab), showing lower response rates, decreased progression free survival and OS compared to patients with *KRAS* wild type tumors.^{13, 14, 30} Therefore, the European Medicines Agency and the Food and Drug Administration have restricted the use of anti-EGFR antibodies in metastatic CRC to patients with *KRAS* wild type tumors. Nevertheless, since only a subset of patients with a *KRAS* wild type tumor respond to treatment with anti-EGFR antibodies, additional predictive markers are needed. The heterogeneity of both *KRAS* wild type and *KRAS* mutated tumors in terms of treatment response has been demonstrated. Within the group of *KRAS* wild type tumors, *BRAF* mutations were shown to have a negative predictive value for anti-EGFR therapy.³¹ However, our group showed that *BRAF* mutations predominantly have a strong negative prognostic value.³² Recently, the negative predictive value of *KRAS* mutations appeared to be limited to patients with *KRAS* codon 12 mutations, while patients with codon 13 mutations were shown to derive comparable benefits from anti-EGFR treatment compared to patients with *KRAS* wild type tumors.³³ These findings warrant further clinical validation. Other *KRAS* mutations (codon 61)³⁴, *PIK3CA* mutations³⁵, PTEN expression³⁶, ligands to the EGFR^{37, 38}, and germline single nucleotide polymorphisms^{39, 40} have shown promising results but need further testing before they can be implemented in routine clinical decision making. In absence of other molecular markers, the predictive strength of the *KRAS* mutation status stresses the importance of RAS-GTPase activity for the response to anti-EGFR therapy. Therefore, other regulatory

mechanisms of RAS-GTPase activity, such as copy number alterations (CNA) of the *KRAS* gene or posttranslational factors (microRNAs) are obvious novel candidate markers (Figure 3).

An additional possible explanation for the failure rate to anti-EGFR antibodies in patients with *KRAS* wild type tumors may be a discordance of *KRAS* mutation status between primary colorectal tumors and their corresponding metastases. *KRAS* mutation status is almost invariably being tested in the primary tumor, since this tissue is usually available as opposed to tissue from metastases. Discordance in *KRAS* mutation status could possibly be explained by heterogeneity of the primary tumor with subsequent progression of one specific subset of tumor cells as a result of clonal selection. Also, late acquirement or loss of *KRAS* mutation during disease progression and the development of metastases could result in a discordance in *KRAS* mutation status. Current data on the concordance in *KRAS* mutation status between primary colorectal tumors and metastases are conflicting.⁴¹⁻⁴³ Therefore, it is still uncertain whether the evaluation of *KRAS* mutation status in the most commonly available primary tumor correctly reflects the *KRAS* mutation status of corresponding metastases. This is highly relevant given the large number of CRC patients that are eligible for anti-EGFR treatment as well as the potential toxicity and costs of this therapy.

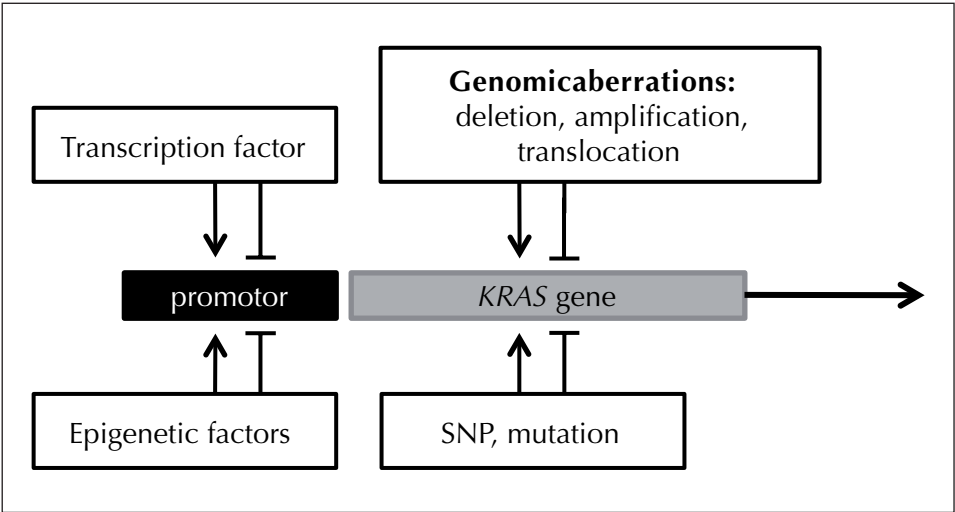


Figure 3. Regulatory mechanisms of RAS-GTPase activity, including transcription factors, genomic aberrations, epigenetic changes, SNPs and mutations. Abbreviation: SNP: single-nucleotide polymorphisms.

THE MOLECULAR PATHWAYS OF COLORECTAL CANCER METASTASES

The formation of metastases is a highly inefficient and complex multistep process, in which malignant cells disseminate from the primary tumor to colonize distant organs. Invasion, which initiates the metastatic process, consists of changes in tumor cell adherence to cells and the extracellular matrix (ECM), proteolytic degradation of surrounding tissue and motility to navigate a tumor cell through tissue. After entering the bloodstream, tumor cells must survive in the circulatory system. These cells extravasate by inducing endothelial retraction, leading to the attachment of tumor cells to the subendothelial ECM and reformation of the capillary. Subsequent steps including proliferation, induction of angiogenesis, and evading apoptotic death are crucial for colonization at the secondary site.⁴⁴ Tumor cells need to be proficient in all these processes in order to produce metastatic outgrowth. Therefore, each step in metastatic outgrowth requires specific genetic and epigenetic changes. One of the challenges in effective detection and treatment lies in the elucidation of these (epi)genetic alterations underlying CRC metastases, which may provide novel targets for future diagnostic tests and treatments.

Genetic instability and colorectal cancer metastases

Chromosomal instability (CIN) is the most common type of genetic instability in CRC, and occurs in approximately 85% of the colorectal carcinomas. CRC carcinogenesis is accompanied by a progressive accumulation of CIN⁴⁵, and these DNA copy number alterations can lead to deregulation of gene expression.⁴⁶ Several chromosomal regions in CRC frequently show gain combined with overexpression (7p, 8q, 13q and 20q) or decreased expression of genes at loci with losses (1p, 4, 5q, 14q, 15q and 18).⁴⁷

In comparison to the many molecular alterations involved in the CRC adenoma-carcinoma sequence, less information is available on the mechanisms responsible for metastases. To gain a better insight in molecular aberrations involved in CRC metastases, one approach is to compare DNA copy number profiles in primary tumors and metastatic tissues. Many studies on this topic have been performed, however in most of these an unmatched-pair approach was used. This type of unmatched comparison is unreliable due to the heterogeneity in endogenous copy number aberrations between primary colorectal carcinomas. In order to overcome this problem, comparison of matched metastatic and primary tumor tissue is essential. Studies including matched-pair data, show a high level of similarity between metastases and their corresponding primary tumors.⁴⁸⁻⁴⁹ Given this close resemblance between primaries and metastases, it was suggested that the capacity to metastasize is largely determined by the mutant alleles that are acquired relatively early during tumorigenesis. A second approach is to compare DNA copy number profiles in primary tumors of patients with and without

metastatic disease, to identify molecular aberrations in primary tumors that are more likely to metastasize. So far, a number of chromosomal aberrations in primary tumors have been associated with the ability to metastasize. Across all studies, gain of chromosome 20q is more frequently observed in primary tumors with liver metastases compared to primary tumors without metastases.⁵⁰ However, a specific comparative genomic hybridization (CGH) profile to predict CRC metastases has not been established.

Microarray analysis is a useful approach for identifying candidate genetic alterations involved in metastases. The relative ease of which pathological specimens can be obtained has facilitated the application of technologies to allow the genome-wide analysis both at the RNA (gene expression) and DNA (aneuploidy) levels. In 1992, Kallioniemi developed chromosome-based CGH, which is a technique used to analyze DNA copy number alterations on a genome-wide scale.⁵¹ Due to its limited resolution, the (micro)array-based CGH was introduced in 1997, which resulted in increased sensitivity.⁵² Briefly, tumor and normal DNA are labelled with green and red fluorochromes, respectively, and hybridized to genomic DNA attached to a slide. Images of the fluorescent signals are captured and the green to red ratios are digitally quantified for each target (Figure 4). These ratios are called into amplifications, gains, losses and no copy number aberrations.⁵³ Array CGH is a powerful molecular cytogenetic method to screen the entire genome for chromosomal imbalances. However, tumor heterogeneity, limited cohort sizes, and methodological differences among experimental and bioinformatic approaches still poses obstacles towards the optimal utilization and integration of genomic profiles.

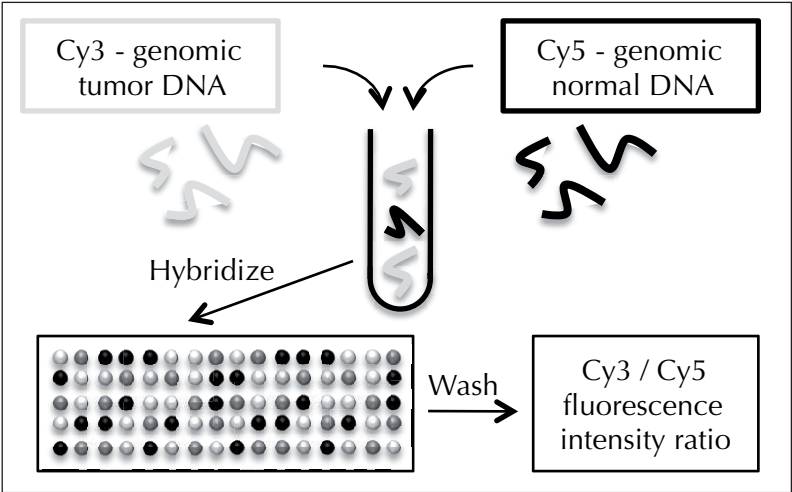


Figure 4. Schematic overview of the microarray CGH technique. Tumor and reference DNA are labeled with Cy3 and Cy5 respectively, and hybridized on a microarray slide containing DNA fragments of the genomes.

Epigenetic alterations and colorectal cancer metastases

In addition to genetic alterations, epigenetic changes such as methylation, histone modification, and post-transcriptional gene regulation by microRNA (miRNAs) also contribute to CRC metastases. Recently, miRNAs have been discovered and are of major interest due to their unique role in cancer development.⁵⁴ MiRNAs are a family of small non-coding RNAs that act as endogenous suppressors of gene expression through imperfect binding the 3'-untranslated region (3'-UTR) of target mRNAs, inducing either translational repression or mRNA degradation (Figure 5). To date, more than 1000 human miRNAs have been identified (miRBase Sequence Database – Release 16) and each individual miRNA may control hundreds of target genes. MiRNAs have important regulatory functions in basic biological processes that form the hallmarks of cancer, such as cellular differentiation, proliferation, migration, and apoptosis. An association between the dysregulation of miRNA expression and specific steps in the metastatic pathway is therefore highly likely. Specific miRNA signatures (miR-21, MiR-17-92, MiR-200 family, miR-196 and MiR-34) have been associated with clinical and biological phenotypes of tumors, and are often dysregulated in CRC.^{55, 56} Alterations in miRNA expression can be caused by various mechanisms, including deletions, amplifications or mutations involving miRNA loci, epigenetic silencing or the dysregulation of transcription factors that target

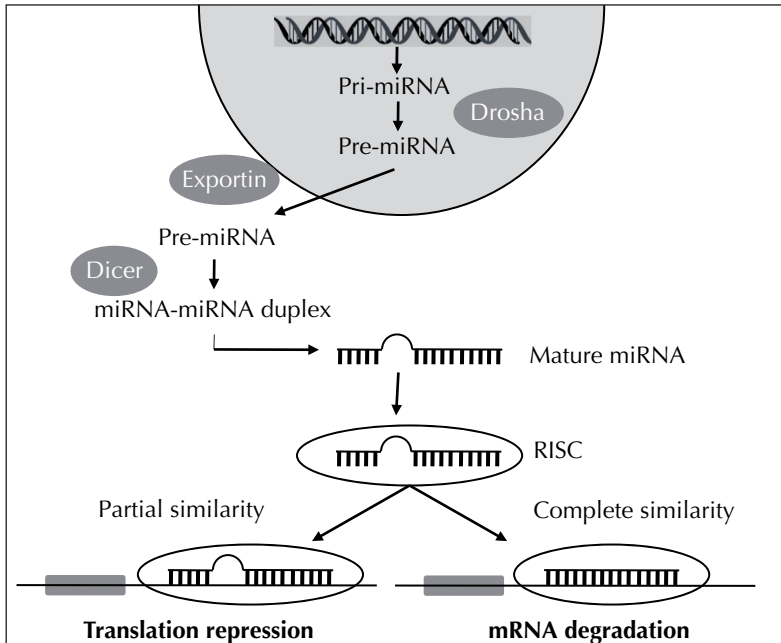


Figure 5. Biogenesis and function of miRNAs.

specific miRNAs. When specific miRNAs involved in metastases are identified, novel therapeutic strategies can be developed.

ORGAN-SPECIFIC METASTASES IN COLORECTAL CANCER

Similar like other types of cancer, CRC metastases show organ preference. The liver is the predominant site in approximately 80% of CRC patients. In 40 to 50% of these patients, extra-hepatic organs are also involved in metastatic colonization. Metastatic cells prefer to grow in certain organs in a way that cannot be explained by circulatory patterns alone. Mechanical entrapment combined with receptor-specific seed- and soil adhesions are currently discussed as determining factors for cancer cell arrest in target organs. Organ specificity has mainly been investigated in mouse models and in vitro assays using CRC cell lines. In these studies it appears that organ-specific formation of CRC metastases is mainly mediated by specific interactions between circulating tumor cells and the vessel wall of potential target organs. The molecular mechanisms underlying these specific adhesive interactions in metastatic target organs remain poorly understood.

The (epi)genomic programmes that determine organ specificity in regard to metastases are probably present at the time when the primary tumor arises. Evidence for this hypothesis comes from the similarity between DNA copy number profiles and mRNA expression profiles between primary tumors and metastases.⁵⁷ Secondly, genomic profiling studies in primary tumors has identified ‘metastases signatures’ that can predict the metastatic phenotype in breast cancer patients.⁵⁸ Identifying genomic regions and genes involved in organ-specific metastases should learn us more about the underlying mechanisms of organ specificity in regard to CRC metastases. This will also help to identify clinically applicable diagnostic markers, preventive approaches, and to find molecules that might be useful targets for therapy.

OUTLINE OF THE THESIS

Metastatic CRC is a common malignancy in the Western World and recent therapeutic advances have prolonged survival of these patients. Nevertheless, the survival rates are very heterogeneous due to the presence of various patient, pathological, and treatment-related prognostic factors. The presence of lymph node metastases is the most powerful prognostic

factor for recurrence and OS in CRC. In **chapter 2** we correlate the amount of lymph nodes retrieved with clinical outcome in rectal cancer patients treated with short-term radiotherapy followed by TME surgery. Furthermore, we evaluated which clinical, primary tumor, and treatment related factors influence lymph node retrieval. The study in **chapter 3** aims to identify the prognostic value of primary tumor histology in metastatic CRC patients treated with the currently used systemic therapy, including targeted agents. In **chapter 4** we present data on a potential prognostic marker in metastatic CRC by comparing clinical outcome between patients with synchronous versus metachronous metastases.

Next to establishing prognostic markers, it is highly relevant to find predictive markers in order to optimize the selection of metastatic CRC patients for specific treatment regimes. The identification of a *KRAS* mutation as predictive marker for the response to anti-EGFR therapy has been one of the most important achievements in the last years. Since only a minority of *KRAS* wild type patients respond to anti-EGFR therapy, it is necessary to find additional predictive markers. We investigated the *KRAS* mutation status and other regulatory mechanisms of *KRAS* in more detail. One possible explanation for the suboptimal response in *KRAS* wild type patients is described in **chapter 5**, where we investigate the level of concordance in *KRAS* mutations status between primary tumors and their corresponding liver metastases. In **chapter 6** we present the correlation of other regulatory mechanisms beyond the *KRAS* point mutation, such as *KRAS* copy number alterations and miRNAs targeting *KRAS*, with response of metastatic CRC patients treated with chemotherapy, bevacizumab and cetuximab.

To develop new therapeutic targets in metastatic CRC, a better insight is required in the (epi)genetic alterations that are responsible for metastases. Recently, miRNAs have been discovered which play a relevant role in cancer progression. In **chapter 7** we review the role of miRNAs in CRC metastases, including escape of apoptosis, epithelial-mesenchymal transition (EMT), angiogenesis, invasion, migration, and proliferation. The presence of additional chromosomal aberrations in CRC metastases compared to matched primary tumors are investigated in a paired approach using array CGH, of which the results are presented in **chapter 8**. In addition to metastases-associated genes in general, an individual primary tumor probably deploys distinct (epi)genetic programs in order to colonize different metastatic sites. Finally, in **chapter 9** we address the issue of metastatic tropism by describing the differences in clinicopathological features and array CGH profiles between primary tumors of CRC patients with hepatic versus extrahepatic metastases.

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**Chapter 2 Lymph node retrieval
in rectal cancer is dependent on
many factors - the role of the
tumor, the patient, the surgeon,
the radiotherapist, and the
pathologist**

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ABSTRACT

Lymph node status is the strongest prognostic factor for survival in colorectal cancer. There are several guidelines concerning the minimum numbers of lymph nodes that need to be examined to make reliable staging possible, but there is no consensus in the available literature. In this study, we determine in patients with rectal cancer factors that relate to the number of lymph nodes found and the presence of lymph node metastasis. In addition, the number of examined lymph nodes was correlated with prognosis.

A total of 1227 patients were selected from a multicenter prospective randomized trial investigating the value of neoadjuvant radiotherapy. The median number of examined lymph nodes in all patients was 7.0. The number of retrieved lymph nodes in patients with node metastasis was significantly higher than in node negative patients. After neoadjuvant radiotherapy fewer lymph nodes were retrieved (6.9 vs. 8.5; $P < 0.0001$). Variations in lymph node yield between pathology laboratories and individual pathologists were striking. The following patient and tumor characteristics are associated with a significant lower lymph node retrieval: age over 60 years, overweight, small size, low invasion depth of the tumor, poor differentiation grade, and absence of a lymphoid reaction. Node negative patients in whom seven or less lymph nodes were examined had a lower recurrence free interval than patients in whom at least 8 lymph nodes were examined (17.0% vs. 10.7%, $P = 0.016$).

We conclude that in pathology laboratories a median of at least 8 lymph nodes need to be examined in rectal cancer specimens, but that higher numbers are desirable and achievable in most cases, even after preoperative radiotherapy.

INTRODUCTION

The presence of metastases in lymph nodes (LN) is the most powerful negative prognostic factor in colorectal cancer and the main indicator for the application of adjuvant therapy. Throughout the years several guidelines have been issued suggesting minimum numbers of LN to be evaluated before patients can be declared node negative, varying from 6 to 17 nodes.¹⁻⁵ In this tumor node metastases (TNM) guidelines, a minimum examination of 12 LN is advised⁶, on the basis of single center studies and data from cancer registries. However, in the era of evidence-based medicine, evidence is preferably gathered using data from well-documented multicenter clinical trials. Data concerning numbers of analyzed LN in clinical trials are scarce, and in general lower than those reported in single center studies. Absence of real evidence in the literature is the reason why the current Dutch guideline advises a minimum examination of 10 LN.⁷ We have shown previously, using the data from a large nation-wide trial for rectal carcinoma, that 12 or more LN are found in only 18% of the cases.⁸ The application of neoadjuvant therapy (short-term radiotherapy (RT) or radio-chemotherapy), now standard care for rectal carcinoma in many European countries, results in even less examined LN.⁹ An adequate nodal staging is difficult because many factors influence the number of LN retrieved. The number of LN found in resection specimens of rectal tumors are significantly less than those in resection specimen of colon tumors.¹⁰ Other factors are related to the patient (sex, weight), the surgeon (specimen size, type of surgical procedure) and to the tumor (size, differentiation, and invasion depth). Pathologic procedures, such as time and type of fixation, have also been shown to be of influence.

The aim of this study was therefore to analyze the influence of several patient, tumor, and treatment related factors on the number of both LN retrieved and the presence of LN metastasis in optimally treated rectal cancer specimens. Furthermore, we correlated the number of examined LN with prognosis. For this analysis data were taken from a large prospective multicenter trial comparing no preoperative treatment with 5 x 5 Gy RT shortly before surgery.

MATERIALS AND METHODS

Study population

Patients were selected from the RT + total mesorectal excision (TME) trial, a large multicenter trial, in which 1530 Dutch patients were included from January 1996 to December 1999.¹¹ This prospectively randomized trial evaluated TME surgery with or without preoperative RT (5 x 5 Gy) for patients with clinical resectable adenocarcinoma of the rectum. Radiotherapeutic, surgical and pathological procedures were described in detail elsewhere.^{8, 12}

Patient selection

For this study we analyzed the data of the eligible Dutch patients in the trial. The following patients were excluded from the analysis: no resection (n=27), resection locally not complete (n=98), distant metastases at operation (n=81), no tumor at operation (n=20). From the RT group those patients who did not receive RT according to protocol: total dose not 25 Gy (n=23), overall treatment time >10 days (n=82), leaving 1227 patients for the analysis. The median follow-up was 68 months.

Pathological procedures

Standardized pathology examination was performed in the pathology laboratories of the referring hospitals using the protocol of Quirke et al.^{13, 14} The specimens were examined for LN, and all found LN were processed for microscopic examination. LN retrieval was performed in a routine matter; no fat clearance methods were applied. The use of the protocol of Quirke et al.^{13, 14} implies a 48-hour formalin fixation and careful slicing of the mesorectal fat, which can contribute to high yields of LN. In a subset of cases the size of the LN was measured on its largest diameter using a grid.

Pathological parameters

Histopathologic classification of tumors was performed using the World Health Organization guidelines.¹⁵ A tumor was considered to be of the mucinous type when at least 50% of the tumor was mucinous. The tumors were graded according to the histological differentiation into well, moderately, poorly, and undifferentiated adenocarcinomas on the basis of the part of poorest differentiation in the tumor. Growth pattern and the presence of inflammatory reaction were assessed according to Jass.¹⁶ The maximum diameter was noted with an invasion depth described in terms of the T classification (TNM).¹⁷

Data collection and statistics

All case record forms were sent to the central data office at the surgery department of the Leiden University Medical Center in Leiden, the Netherlands. The data were checked and entered in a database and analyzed with the SPSS package (SPSS 14.0 for Windows, SPSS Inc, Chicago, IL). Relations between various parameters were analyzed by using χ^2 test, Mann-Whitney U test, and Kruskal-Wallis nonparametric testing procedures. Multivariate analysis was performed using the enter method in logistic regression model. Univariate survival analyses of time to overall recurrence free survival were performed using the Kaplan-Meier method, with the time of surgery as the entry date. Differences in observed survival between groups were tested for statistical significance using log-ranks tests. A P value of ≤ 0.05 was considered statistically significant.

RESULTS

Patient population

One thousand two hundred twenty seven patients (782 male, 63.9%) with a median age of 64.1 years at randomization (range: 23 to 92 years) were analyzed in this study. All patients underwent a potentially curative operation and 46.1% (n=564) received short-term preoperative RT.

Pathologic examination showed that most tumors were T3 (57.8%). The majority of tumors were adenocarcinomas (89.6%), within 67.2% a moderate differentiation, and in 26.9% a poor differentiation.

A total number of 10748 LN was examined, varying from 0 to 52 LN per patient (median 7.0, quartile range: 4.0 to 12.0). Of all patients, 38.8% (n=474) presented with LN metastases (N+), the remaining 61.2% (n=749) presented without LN metastases (N0).

Factors influencing lymph node retrieval

In N0 patients the mean number of LN examined was 7.7 compared with 10.5 in N+ patients ($P < 0.001$). Because of this finding, the different factors, which influence lymph node retrieval were separately analyzed in patients with positive and negative LN, to avoid bias because of the effects of lymph node status.

Treatment-related factors

Radiotherapy

In the RT group of N0 patients the mean number of examined nodes was 6.9 versus 8.5 in the surgery only group ($P<0.0001$). This significant difference was also present in node positive patients (Table 1).

In series of 258 LN (28 patients) from 1 of the participating hospitals, we evaluated the effects of RT on the size of the examined LN. LN were somewhat smaller after RT (mean size 12.6 mm² in TME vs. 10.8 mm² in RT), however, this difference was only present in the LN with metastases. The size of the uninvolved LN was 9.9 mm² in both groups (Figure 1).

Surgical procedures

The type of resection, being directly related to the location of the tumor, was strongly correlated with the number of LN in N0 patients. Lymph node retrieval after low anterior resection was 8.7, versus 6.0 after abdominoperineal resection. This difference was not observed in N+ patients (Table 1).

However, contrary to expectations, when longer parts of bowel were removed, the number of examined LN did not increase. Especially in the N0 group, there seemed to be almost an inverse relation between length of specimen and number of LN, that is, 7.8 in specimen ≤ 20 cm vs. 5.8 in specimen >40 cm ($P=0.06$) (Table 1).

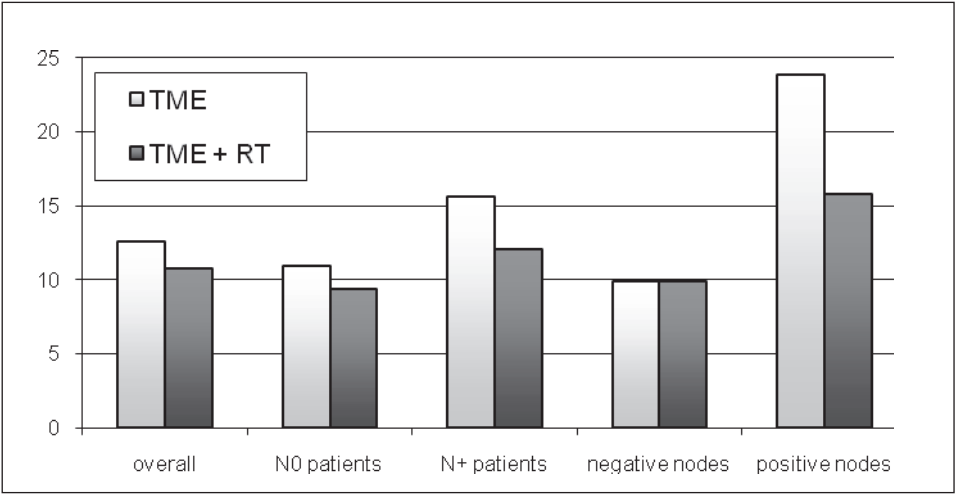


Figure 1. The mean LN size (mm²) in relation to the randomization arm. The first column shows the overall mean LN size. In the second and third column the mean LN sizes are shown for N0 and N+ patients, respectively. The fourth and fifth column show the mean sizes of LN that, respectively, do not and do contain metastases.

Pathological procedures

Substantial variance was found between the 49 participating pathology laboratories (Kruskal-Wallis $P < 0.001$), with median (quartile range, number of specimens) yields varying between 1.5 (0.75-4.3, $n=6$) and 13.5 (4.5-24.8, $n=4$) (Figure 2). The number of specimens per laboratory varied between 2 and 120, but this was not associated with the median LN yield. In Figure 2 individual scores per pathologist are shown for 3 selected laboratories. Only data from nonirradiated patients were used as it is likely to find more LN in this subset, as described above. From this figure can be deduced that large differences exist between various pathologists and between laboratories, median yields vary from 3.5 (laboratory B) to 17 (laboratory C).

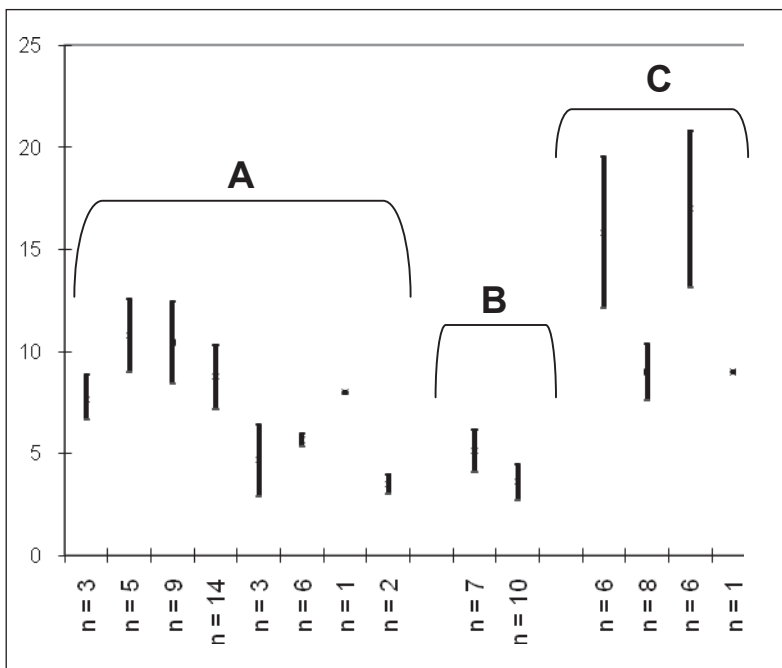


Figure 2. Number of examined lymph nodes in node-negative patients in 3 different pathology laboratories. The first one (**A**) is a large laboratory with a median number of 7.0 (quartile range 5.5 to 11.0) LN per specimen. The second laboratory (**B**) with 2 pathologists reached a median number of 4.0 (quartile range 1.75 to 6.0) LN per specimen. The third laboratory (**C**) recovered the highest median number of lymph nodes in the Netherlands (median 13.0, quartile range 9.0 to 17.0). On the x-axis the number of specimen per pathologist is given. Numbers of lymph nodes are given as median \pm quartile range.

Table 1. Factors influencing mean lymph node retrieval in node positive and negative patients (univariate analysis)

		Node negative			Node positive		
		Mean LN	N	P value	Mean LN	N	P value
Treatment characteristics							
Randomization	TME	8.5	392	0.000*	11.6	269	0.000*
	TME + RT	6.9	360		9.1	204	
OK type	LAR	8.7	462	0.001*	10.5	309	0.693*
	APR	6.0	234		10.5	135	
Quality of mesorectum	Good/moderate	8.5	364	0.060*	10.4	245	0.294*
	Poor	6.7	166		9.8	96	
CRM	≤ 2 mm	8.3	110	0.529†	10.7	135	0.836†
	2 - 5 mm	7.5	160		10.3	104	
	> 5 mm	7.7	481		10.5	234	
Distal margin	≤ 2 mm	8.0	285	0.026†	10.6	131	0.713†
	2 - 5 mm	7.2	303		10.6	212	
	> 5 mm	8.6	118		10.6	71	
Length of specimen	≤ 20 cm	7.8	151	0.060†	9.9	95	0.683†
	20 - 30 cm	7.8	344		10.2	219	
	31 - 40 cm	7.5	139		10.5	92	
	> 40 cm	5.8	41		12.2	20	
Patient characteristics							
Sex	Male	7.8	489	0.156*	10.5	296	0.873*
	Female	7.5	263		10.5	177	
Age	< 60 years	9.0	247	0.001*	10.9	182	0.342*
	> 60 years	7.1	505		10.2	291	
BMI	≤ 20	10.4	25	0.021†	9.6	20	0.155†
	20 - 25	8.3	238		10.7	171	
	> 25	7.4	322		9.3	178	
Weight loss	Yes	8.2	218	0.075*	10.7	146	0.260*
	No	7.5	498		10.3	310	
Tumor characteristics							
Histological type	Adenocarcinoma	7.8	689	0.298*	10.3	407	0.196*
	Mucinous carcinoma	6.9	60		11.3	62	
Invasion depth	T1	5.3	63	0.001†	8.5	2	0.563†
	T2	7.1	322		10.5	92	
	T3	8.6	352		10.3	356	
	T4	9.7	15		13.7	23	
Size	≤ 2 cm	4.4	62	0.000†	7.4	21	0.000†
	2 - 5 cm	7.3	502		9.8	329	
	> 5 cm	10.0	181		13.1	114	
Differentiation	Well/moderate	8.0	584	0.004*	10.5	303	0.933*
	Poor/undifferentiated	6.6	167		10.4	169	
Lymphoid reaction	None/few	7.5	631	0.014*	10.5	430	0.831*
	Extensive	8.8	117		10.4	43	

Abbreviations: BMI = body mass index; LN = lymph node; RT = radiotherapy;
 APR = abdominoperineal resection; LAR = low anterior resection;
 TME = total mesorectal excision; CRM = circumferential resection marge
 * Mann Whitney U test † Kruskal-Wallis test

Patient-related factors

The number of examined LN was significantly smaller in N0 patients of 60 years and older (9.0 vs. 7.1, $P<0.001$). There was no effect of sex. In overweighted N0 patients (BMI >25) less LN were examined compared with patients with normal weight or underweight (7.4 vs. 8.3 and 10.4, respectively, $P=0.021$). None of the patient related factors had a significant effect on lymph node retrieval in N+ patients (Table 1).

Tumor-related factors

In specimens with larger tumors more LN were found, 10.0 in tumors over 5 cm in diameter, compared with 4.4 in tumors less than 2 cm in size in N0 patients ($P<0.0001$). This significant difference was also present in N+ patients. This was also reflected in the relation of the number of LN with invasion depth. Well-differentiated tumors showed more LN in N0 patients (8.0 vs. 6.6, $P=0.004$). In N0 patients the retrieval of LN was increased in tumors with an extensive lymphoid reaction (8.8 vs. 7.5, $P=0.014$). Histological type did not influence the numbers of LN (Table 1).

Table 2. Multivariate analysis in node negative patients for at least 12 examined lymph nodes

Tumor, treatment, and patient characteristics		OR	95% CI	P value
Randomization	TME + RT	0.8	0.5 - 1.2	0.22
	TME	1.0		
OK type	LAR	2.1	1.4 - 4.0	0.017
	APR	1.0		
Age	< 60 years	1.7	1.2 – 2.6	0.007
	> 60 years	1.0		
Invasion depth	T1	0.3	0.1 – 0.5	0.13
	T2	0.5	0.1 – 1.9	0.31
	T3	0.7	0.2 – 2.7	0.65
	T4	1.0		
Size	≤ 2 cm	0.3	0.1 – 0.9	0.027
	2 - 5 cm	0.6	0.4 – 0.9	0.022
	> 5 cm	1.0		
Differentiation	Well/moderate	2.1	1.0 – 2.8	0.061
	Poor/undifferentiated	1.0		

Factors are included in the multivariate analysis if their p -value was < 0.05 in the univariate analysis. Abbreviations: APR = abdominoperineal resection; CI = confidence interval; LAR = low anterior resection; RT = radiotherapy; TME = total mesorectal excision

Multivariate analysis in node negative patients

In the multivariate analysis we included all factors showing a significant correlation with the number of LN in the univariate analyses in node negative patients. The cut-off value of 12 LN was chosen on the basis of current TNM guidelines.²³ In these analyses only the age of the patient, the size of the tumor, and the surgical procedure showed a significant relation with the number of LN (Table 2).

Factors associated with the presence of lymph node metastases

Younger patients (<60 years) are more often diagnosed with N+ status compared with older patients, however, this difference was not significant ($P=0.051$). None of the other patient related factors were associated with lymph node status. Short-term neoadjuvant RT did not affect nodal status ($P=0.10$). Of all the surgical related factors only distal and circumferential margin involvement were associated with N+ status ($P=0.007$ and $P<0.0001$, respectively), furthermore the tumor was located closer to the circumferential margin in N+ patients ($P<0.0001$).

Almost all morphology-assessed features were associated with LN status. N+ status was positively associated with larger tumor diameter ($P=0.038$), higher T-stage ($P<0.0001$), mucinous carcinoma ($P=0.002$), poor differentiation ($P<0.0001$) and absence of/or little lymphoid reaction ($P=0.001$) (data not shown).

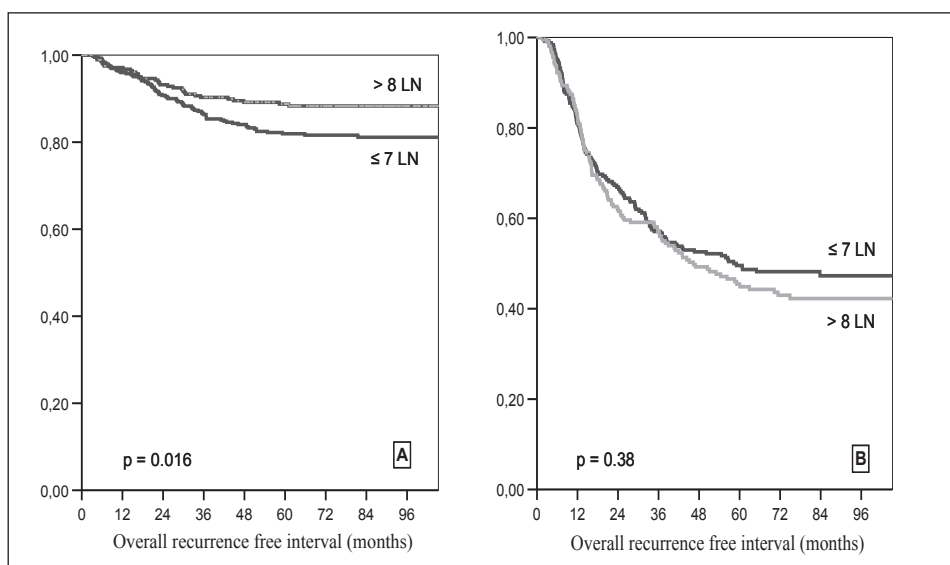


Figure 3. Overall recurrence free interval on the basis of median LN yields per pathology laboratory. A, N0 patients. B, N+ patients.

Lymph node status and retrieval in correlation with survival

To evaluate the impact of a low number of LN examined on patients survival we divided the participating pathology laboratories into 2 groups, the ones with a high median number of examined LN (>8, 48 laboratories, 513 patients) and those with a low median number of examined LN (<7, 48 laboratories, 711 patients). These cut-off value was determined using the median lymph node numbers in the RT arm of our trial and by various studies in the literature.^{3,25} When evaluating prognosis, we found that N0 patients from low LN laboratories have a significantly lower recurrence free interval (17.0% vs. 10.7%, $P=0.016$, Figure 3A). There was no difference in prognosis for N+ patients (50.6% vs. 46.7%; $P=0.38$, Figure 3B). This suggests that the LN status is more reliable if it is determined in a laboratory with high LN yields.

DISCUSSION

The presence of LN metastases is the most powerful prognostic factor for recurrence and overall survival in colorectal cancer. In this study, we evaluated factors involved in the amount of both examined LN and lymph node status in rectal cancer patients. Furthermore, we demonstrated that the amount of LN retrieved is correlated with clinical outcome.

The number of patients in our study with 12 or more LN was low, only 19% of patients who received preoperative RT and 31% of patients that underwent surgery only. These percentages are similar to those found by Baxter et al.¹⁸ in a large retrospective study on the impact of preoperative RT on LN retrieval in rectal cancer. Tepper et al.⁵ conducted a study in 1664 rectal cancer patients that underwent TME surgery and subsequently RT and 5-FU-based chemotherapy. The quartile ranges of the number of LN examined in this study were similar to our TME only group.

The number of 12 LN is not based upon large-scale support, so continuously data is being generated and calculations are being made, attempting to find clinically relevant cut-off points. This has resulted in diverging recommendations, from 6 to 16 LN per colorectal resection specimen.^{1-4, 10, 19, 20}

We identified several treatment, patient and tumor related factors that influence the number of retrieved LN. Because LN status is an important factor involved in the amount of examined LN, we analyzed all factors in these 2 groups separately. Compared with surgery alone, the application of neoadjuvant RT seriously interferes with the detection of LN and results in lower LN yields. In LN with metastases the mean size of the node is smaller because the lymphoid cells disappear by radiation. This phenomenon is not seen in negative LN, most likely because

the shrinking negative LN become so small that they are under the level of detection after neoadjuvant therapy.

Not only neoadjuvant therapy is affecting the number of LN retrieved. Indeed, surgical procedures play a part too. One can imagine that when TME surgery has been carried out adequately, more fatty tissue will be removed and as a consequence more LN can potentially be found. Indeed, in cases where the surgical quality of the resection specimen was good (i.e. resection margin on the mesorectal fascia), more LN were found. One would expect that more LN could be found, if a longer segment of bowel is removed. In contrast, the longer the resection specimen, the less LN (per 10 cm) were found, although this did not reach the significance. These results partly can be explained by anatomy, as LN are not equally distributed along the bowel.²¹⁻²⁴ The majority of mesorectal LN were located within the upper two-thirds of the posterior mesorectum.²² Complete removal of nodes in this area may, in part, explain higher yield of LN in a smaller resection specimen.

Pathology laboratories showed large differences of the median amount of examined LN. The International Union against Cancer guidelines advise to examine at least 12 LN per specimen for accurate N-staging. This number is arbitrary, however, and in this study we found that this number is only met in the upper quartile of the study population. In the lowest quartile, no more than 4 LN were examined per specimen, a result obtained even after special training of pathologists. In this RT+TME trial the pathologic examination of the specimen was standardized and pathologists were trained according to the protocol of Quirke et al.¹³ Nevertheless, for the examination of most specimens the criteria of the International Union against Cancer minimum LN yield were not met. It is speculative to precisely indicate the underlying explanations for these variations, but it is plausible that in spite of training, working standards still did not meet the minimal demands in some laboratories. In contrast to most studies, derived from single institutions, we believe that our findings reflect routine practice better, as was confirmed by the data from the Surveillance, Epidemiology and End Results program database.²⁵ This is an important issue, as we found that prognosis is more adequately predicted for N0 patients in laboratories where at least 8 LN were retrieved. In laboratories where routinely 7 or less LN was examined, N0 patients have a lower recurrence free interval. This confirms that low LN yields leads to the understaging as positive LN are not retrieved, thereby excluding patients from adjuvant therapy, probably resulting in a poor prognosis.²⁶

Understaging because of low LN yields is not only attributable to the pathologist, patient and tumor related factors are of significant importance too. Biologic difference in immune response may lead to more prominent LN, which makes them easier to identify. The amount of inflammatory response is dependent on the age of the patient, size, and invasion depth of the tumor.²⁷

Most of the studies concerning lymph node counts were performed in a mixture of colorectal cancer patients, like the studies of Caplin et al.²⁸ (at least 7 LN should be analyzed) and Wong

et al.¹⁰ (at least 14 LN should be analyzed). These numbers cannot simply be extrapolated for rectum carcinomas, as several previous studies report that the mean LN in rectum is lower compared with other colon segments.²¹⁻²⁴

In a large single center study of 2427 pT3 colorectal adenocarcinomas, Goldstein et al.²⁶ showed that, increasing numbers of recovered LN were associated with a greater number of LN metastases and specimen without LN metastases had lower mean LN yields. During the 45 years of this study, the standard dissection of LN did not change much, but before 1992 LN dissections were performed by pathologists and residents, and after 1992 this was performed by trained pathology assistants. This switch led to a striking increase of yearly means of recovered LN, suggesting a crucial role for the person that is performing the dissection. Thorn et al.²⁹ confirmed this in their search for factors that affect LN yield in surgery for rectal cancer. They found that besides tumor size and number of positive LN, the examining pathologist was of independent significance in multivariate analysis. Our data confirm this finding.

Conventional manual node dissection is accurate and efficient but time-consuming. We were able to show that in a number of pathology laboratories the median of 12 LN per patient is achieved with conventional methods, the number suggested as minimum by the TNM-system for both colon and rectal cancer, thereby demonstrating its feasibility, however, this was achieved in only a minority of all specimens.

Several studies have reported about additional techniques to improve the N-staging by increasing the number of detected LN, using techniques like sentinel node procedures, fat clearing methods, and ex vivo methylene blue injection.³⁰ However, studies are small, clinical relevance is unclear and reliability is a major issue. Therefore, as long as the clinical consequences of these procedures have not been confirmed indisputably, there is a limited need for additional methods and standard evaluation of LN should be sufficient.

In conclusion, we showed that the number of retrieved LN after TME surgery is partially dependent on biologic characteristics, and is partially influenced by the choice of treatment and pathologic assessment of the specimen. Standard evaluation of LN should be sufficient if standard pathologic procedures are carried out thoroughly and accurately. The potential additional benefit of immunohistochemical or sentinel procedures can only thereafter be assessed. The International Union against Cancer guidelines advise to examine at least 12 LN per specimen for accurate N-staging, both for colon and rectal cancer. This number is arbitrary, however, and in this study we found that this number is only met in the upper quartile of the study population. In the lowest quartile, no more than 4 LN were examined per specimen. Our data indicate that a median of 8 or more LN indicate that adequate staging is performed in patients with rectal cancer.

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Chapter 3 **Mucinous adenocarcinomas: poor prognosis in metastatic colorectal cancer**

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ABSTRACT

PURPOSE: Mucinous histology of metastatic colorectal cancer (CRC) has been associated with poor prognosis, however this has never been assessed in large well-defined study populations treated with the current used systemic agents. We investigated the prognostic value of mucinous histology in two large phase III studies in metastatic CRC.

PATIENTS AND METHODS: The study population included 1010 metastatic CRC patients who were treated with chemotherapy and targeted therapies in two phase III studies. Patients were classified according to the histology of the primary tumor in mucinous adenocarcinomas (MC) and non-mucinous adenocarcinomas (AC).

RESULTS: Patients with MC (n=99) were older, had more often a normal serum lactate dehydrogenase (LDH), extrahepatic localization of metastases, larger primary tumor diameter and a higher T classification compared to patients with AC (n=911). A deficient mismatch repair system and *BRAF* mutations were observed in 17% and 22% of patients with MC, compared to 3% and 7% in patients with AC, respectively. Clinical outcome was investigated in both studies separately, showing a worse overall survival (OS), progression free survival, and overall response rate in patients with MC compared to patients with AC. Patients with MC received less cycles of treatment compared to AC, but did not suffer from a higher incidence of grade 3/4 toxicity. In multivariate analysis, mucinous histology was as an independent negative prognostic factor for OS, resulting in a combined hazard ratio of 1.78 (95%CI 1.35-2.35).

CONCLUSIONS: Patients with metastatic mucinous CRC have distinct clinicopathological features and poor response to chemotherapy and targeted agents. The strong negative prognostic value of MC warrants the use of this pathological feature as a stratification factor for clinical trials in metastatic CRC.

INTRODUCTION

Mucinous adenocarcinomas (MC) are a histological subtype of colorectal cancer (CRC), in which the tumor cells secrete abundant extracellular mucin involving more than 50% of the tumor volume. They account for 5-15% of all primary colorectal carcinomas¹, and MC are clinically, morphologically, and molecularly different from adenocarcinomas (AC). MC are more often correlated with an advanced stage at presentation^{2,3}, a larger diameter⁴, localization in the proximal colon^{2,4}, and peritoneal dissemination.⁵⁻⁷ Furthermore, MC affect younger patients and are associated with the Lynch syndrome.^{8,9} Mucinous tumors exhibit a distinct molecular profile compared to AC, involving a higher incidence of *KRAS* and *BRAF* mutations^{10,11}, diploidy¹², deficient mismatch repair system (dMMR)¹³, and CpG island hypermethylation.¹⁴ Approximately 50% of CRC patients develop metastatic disease and in case of irresectable metastases there is no curative treatment available. However, survival can be improved by using cytotoxic regimens (fluoropyrimidines, oxaliplatin, irinotecan) in combination with targeted therapy (VEGF- and EGFR antibodies). Two studies investigated the response to chemotherapy in advanced CRC patients with MC versus AC. Mucinous tumors appeared to be less responsive to fluoropyrimidines⁶, irinotecan and oxaliplatin-based chemotherapy.⁷ The response of tumors with mucinous histology to chemotherapy plus targeted agents has not been reported.

The prognostic value of mucinous histology remains controversial. Several studies report that MC are associated with poor prognosis^{6,7,15}, which however is not confirmed by others.^{2,16} This may be attributed to the small number of patients with MC in these studies. It should be noted that several known prognostic factors, like TNM classification and *BRAF* mutation status¹⁷, were not included in most of these studies.

Therefore, the aim of this retrospective analysis was to assess the prognostic value of MC in metastatic CRC in more detail.

PATIENTS AND METHODS

Study population

All patients included in our analysis participated in two phase III randomized clinical trials from the Dutch Colorectal Cancer Group (DCCG). In the CAIRO study¹⁸ (CKTO 2002-07, ClinicalTrials.gov; NCT00312000) 820 metastatic CRC patients were randomized between first-line sequential or combination treatment with capecitabine, irinotecan, and oxaliplatin. The primary endpoint was overall survival (OS), and secondary endpoints included progression free survival (PFS) and response rate. Median OS was 16.3 months (95% CI 14.3-18.1) for the sequential treatment group and 17.4 months (95% CI 15.2-19.2) for the combination group ($p=0.33$). As expected, combination therapy was associated with a prolonged first-line PFS compared with sequential therapy (7.8 vs 5.8 months; $p=0.0002$). The CAIRO2 study¹⁹ (CKTO 2005-02, ClinTrials.gov; NCT00208546) included 755 metastatic CRC patients, who were randomly assigned to receive first-line treatment with capecitabine, oxaliplatin, and bevacizumab, or the same schedule with the addition of cetuximab. The primary endpoint of this study was PFS, and secondary endpoints were OS and response rate. The addition of cetuximab significantly decreased the median PFS (9.6 months (95% CI 8.4-10.5) versus 10.7 months (95% CI 9.7-12.3); $p=0.01$). The median OS was 20.3 months (95% CI 17.8-24.7) in the patients treated without cetuximab and 19.4 months (95% CI 17.5-21.4) in the patients treated with cetuximab ($p=0.16$). In both studies, treatment cycles were given every 3 weeks. Assessment of the tumor response was performed every 3 cycles (9 weeks) using CT imaging and evaluated according to the Response Evaluation Criteria for Solid Tumors (RECIST).²⁰ The written informed consent required for all patients before study entry also included translational research on tumor tissue.

In our analysis, we included eligible patients with a resection of the primary tumor of which formalin-fixed paraffin-embedded (FFPE) tissue was available. The patients were classified based on the histology of the primary tumor according to the guidelines of the World Health Organization (WHO).²¹ Tumors were considered MC if more than 50% of their volume consisted of mucin. AC were defined as tumors without extracellular mucin. Other histological subtypes were excluded from our analysis.

Clinicopathological features

The following clinical parameters were collected for each patient: age, gender, Eastern Cooperative Oncology Group (ECOG) performance status, serum lactate dehydrogenase (LDH), site of the primary tumor, prior adjuvant therapy, number and sites of metastatic

disease, metachronous (>6 months after initial diagnosis) or synchronous (≤ 6 months of initial diagnosis) presentation of metastases, and regimen used as first-line treatment.

Histopathological review was performed on haematoxylin and eosin-stained coupes by two independent observers, including an experienced pathologist (IDN). The TNM classification of malignant tumors was used to describe the extent of cancer spread in terms of invasion depth and lymph node stage.²² The maximum diameter, median number of detected and involved lymph nodes were recorded.

Microsatellite instability

Immunohistochemistry (IHC) was performed on tissue microarrays (TMA's) of FFPE material of the primary tumor. To determine the expression of the four mismatch repair (MMR) proteins (MLH1, MSH2, MSH6 and PMS2) the slides were stained with antibodies against these proteins, as described before.²³ Tumors were considered positive for MMR expression if nuclear staining was present in at least one tumor cell and negative if there was complete absence of nuclear staining.

Analysis of microsatellite instability (MSI) was performed in all tumors of which at least one staining for the MMR proteins was negative or the IHC staining was not interpretable, using a validated protocol.²³ Two microsatellite markers (BAT 25 en BAT 26) were used, and if only one of these markers showed instability, the analysis was extended with four other markers (BAT 40, D2S123, D5S346, D17S250). A tumor was defined MSI if at least two of the six markers showed instability.

Hypermethylation of *MLH1* promoter

The DNA methylation status of the *MLH1* promoter regions was determined after bisulphite treatment of the DNA using the EZ DNA methylation KIT, ZYMO Research (Orange, CA, United States of America (USA)) as described before.²⁴

KRAS and *BRAF* mutation analysis

Genomic DNA was isolated from 4-8 microdissected 50 μ m sections of FFPE primary tumor tissue as previously described.²⁵ The *KRAS* and *BRAF* mutation status^{25, 26} were assessed in duplicate by sequencing analysis in patients of the CAIRO2 study.

Statistical analysis

The comparison in baseline clinicopathological features between MC and AC was done regardless of study treatment, using the χ^2 -test, Fisher's Exact Test or Wilcoxon rank sum test where appropriate. Clinical outcome of patients with MC and AC was investigated in both studies separate, due to an improvement of clinical outcome caused by the addition of bevacizumab to first-line chemotherapy in the CAIRO2 study. OS was calculated as the interval from the date of randomization until death from any cause or until the date of last follow-up. PFS for first-line treatment was calculated from the date of randomization to the first observation of disease progression, death from any cause or last follow-up date. OS and PFS curves were estimated using the Kaplan-Meier method and compared with the log-rank test. Overall response was defined as partial response or complete response. Disease control was defined by stable disease with a duration of more than four months or partial response or complete response. Differences in response and disease control rates were tested with a χ^2 -test. Multivariate analysis of OS was performed by means of a Cox proportional hazard model to determine if mucinous histology was an independent prognostic factor for survival after correction for; age, gender, localization of metastases, performance status, serum LDH, site of primary tumor, prior adjuvant therapy, metastatic sites involved, onset of metastases, treatment arm, invasion depth, and lymph node status. In the CAIRO2 study, *KRAS* and *BRAF* mutation status were also included in the multivariate analysis. The adjusted hazard ratios of mucinous histology in the CAIRO and CAIRO2 study were checked for heterogeneity. If no heterogeneity was present, the hazard ratios were combined on a log scale, using a DerSimonian-Laird random effects meta-analysis with inverse variance weighting. *P* values below 0.05 were considered as statistically significant. The analyses were performed using SAS 8.2 software.

RESULTS

Study population

A total of 1099 eligible patients, 552 from the CAIRO and 547 from the CAIRO2 study were available for our analysis. In the CAIRO study, 50 patients (9%) were classified with MC, 435 patients (79%) with AC, and 67 patients (12%) with other histological subtypes, including adenocarcinomas with a mucinous component of less than 50%. In the CAIRO2 study, 49 patients (9%) were diagnosed with MC, 476 patients (87%) with AC, and 22 patients (4%) with other histological subtypes of CRC. Patients with other histological subtypes were excluded from our analysis.

Table 1. Baseline characteristics of patients with MC and AC treated in the CAIRO and CAIRO2 study

		MC n = 99	AC n = 911	p-Value
Age	Median (range)	67 (36-84)	63 (28-81)	0.005
Gender	Male	58 (59%)	548 (60%)	0.83
	Female	41 (41%)	363 (40%)	
Localization metastases	Hepatic	19 (20%)	303 (34%)	0.02
	Extrahepatic	40 (41%)	281 (31%)	
	Hepatic+extrahepatic	38 (39%)	312 (34%)	
	Locally advanced	0	7 (1%)	
	Unknown	2	8	
WHO performance status	0	66 (67%)	594 (65%)	0.61
	1	30 (30%)	301 (33%)	
	2	3 (3%)	16 (2%)	
Serum LDH	Normal	80 (82%)	557 (62%)	<0.0001
	>ULN	18 (18%)	347 (38%)	
	Unknown	1	7	
Site of primary tumor	Colon	52 (53%)	418 (46%)	0.08
	Rectosigmoid	30 (30%)	240 (26%)	
	Rectum	17 (17%)	252 (28%)	
	Unknown	0	1	
Prior adjuvant therapy	No	84 (85%)	758 (83%)	0.69
	Yes	15 (15%)	152 (17%)	
	Unknown	0	1	
Metastatic sites involved	1	41 (42%)	420 (47%)	0.47
	2	34 (35%)	316 (35%)	
	>2	22 (23%)	160 (18%)	
	Unknown	2	15	
Metastases onset	Metachronous	46 (46%)	411 (45%)	1.00
	Synchronous	53 (54%)	500 (55%)	
Treatment arm CAIRO study	Sequential	26 (52%)	212 (49%)	0.77
	Combination	24 (48%)	223 (51%)	
Treatment arm CAIRO2 study	CAPOX + Bev	32 (65%)	231 (49%)	0.03
	CAPOX + Bev + Cet	17 (35%)	245 (51%)	

Abbreviations: ULN = upper limit of normal; CAPOX = capecitabine and oxaliplatin; Bev = bevacizumab; Cet = cetuximab; MC = mucinous adenocarcinoma; AC = adenocarcinoma

Patient characteristics

The median age of patients with MC (n=99) was 67 years compared to 63 years for patients with AC (n=911)(p=0.005). Patients with MC more often had a normal serum LDH (p<0.0001), and extrahepatic localization of metastases (p=0.02) compared to patients with AC. A trend was observed for a decreased incidence of MC versus AC in rectum carcinomas (p=0.08). In the CAIRO2 study, a lower percentage of patients with MC was treated with cetuximab (p=0.03) (Table 1).

Table 2. Primary tumor characteristics of patients with MC and AC treated in the CAIRO and CAIRO2 study

		MC n = 99	AC n = 911	p-Value
Diameter (mm)	Median (range)	50 (20-140)	40 (3-135)	<0.0001
Invasion depth	T 1-2	3 (3%)	71 (8%)	0.03
	T 3	64 (67%)	632 (72%)	
	T 4	28 (30%)	172 (20%)	
	Unknown	4	36	
Lymph node status	N 0	24 (26%)	246 (29%)	0.20
	N 1	27 (30%)	307 (36%)	
	N 2	40 (44%)	294 (35%)	
	Unknown	8	64	
Number lymph nodes	Median (range)	9 (1-55)	8 (0-44)	0.23
Number positive lymph nodes	Median (range)	3 (0-54)	2 (0-41)	0.06
MMR status	pMMR	82 (83%)	884 (97%)	<0.0001
	dMMR	17 (17%)	27 (3%)	
KRAS mutation status (CAIRO2 study)	Wild-type	27 (59%)	275 (61%)	0.75
	Mutation	19 (41%)	176 (39%)	
	Unknown	3	25	
BRAF mutation status (CAIRO2 study)	Wild-type	35 (78%)	421 (93%)	0.002
	Mutation	10 (22%)	30 (7%)	
	Unknown	4	25	

Abbreviations: MMR = mismatch repair genes; pMMR = proficient MMR; dMMR = deficient MMR; MC = mucinous adenocarcinoma; AC = adenocarcinoma

Primary tumor characteristics

The primary tumor diameter of patients with MC (n=99) was significantly higher compared to primary tumors of AC patients (n=911)($p<0.0001$). Tumors with mucinous histology had a higher T classification in comparison with AC ($p=0.03$). Furthermore, a trend was observed towards a higher median number of positive lymph nodes in patients with MC compared to AC ($p=0.06$). dMMR was observed in 17% of the mucinous primary tumors compared to 3% in AC ($p<0.0001$). Hypermethylation of the *MLH1* promoter was the cause of dMMR in 83% of the tumors with mucinous histology. In the CAIRO2 study, *BRAF* mutations were demonstrated in 22% of the MC patients compared to 7% in AC patients ($p=0.002$) (Table 2).

Outcome of treatment with chemotherapy (CAIRO study)

Patients in the CAIRO study were treated with first-line sequential or combination chemotherapy containing capecitabine, irinotecan and oxaliplatin. In univariate analysis, the median OS for patients with MC was 13.2 (95%CI 10.2-17.9) compared to 19.2 months (95%CI 17.9-20.6) in the group with AC ($p=0.03$). A trend of decreased PFS was observed in patients with MC versus AC ($p=0.09$). These differences in OS and PFS were observed in both treatment arms. The overall response rate in the 437 patients who were evaluated was 12% in the MC group and 37% in the group patients with AC ($p=0.0006$). Disease control was observed in 79% of the patients in the MC group and 87% of those in the AC group ($p=0.17$). The median number of the overall treatment cycles was significantly different between patients with MC versus AC (6 versus 12 respectively, $p=0.004$). The reasons for treatment discontinuation were not significantly different between both patients groups. The incidence of any grade 3 or 4 adverse event was 72% in the patients with mucinous histology and 66% in the AC patients ($p=0.43$) (Table 3).

Outcome of treatment with chemotherapy plus targeted agents (CAIRO2 study)

In the CAIRO2 study patients received first-line capecitabine, oxaliplatin and bevacizumab with or without cetuximab. The median OS was significantly lower for patients with MC compared to patients with AC (median 13.1 (95%CI 9.9-20.2) versus 21.5 months (95%CI 19.8-22.9); $p=0.009$). Furthermore, the median PFS in the MC group was 7.2 months (95%CI 5.7-8.6) compared to 10.6 months (95%CI 10.1-11.4) in patients with AC ($p<0.0001$). These differences in OS and PFS were observed in both treatment arms. The overall response rate for the patients with MC was 10%, and 54% for the group with AC ($p<0.0001$). In 85% of the MC patients disease control was observed compared to 94% of the patients with AC ($p=0.003$). Patients with MC received less cycles of treatment compared to patients with AC (6 versus 9

cycles, $p=0.006$). No significant differences were observed in the causes for discontinuation of treatment between the group with MC and the group with AC. The incidence of any grade 3 or 4 adverse event was 71% versus 79% in the MC and AC patients ($p=0.27$) (Table 4).

The independent prognostic role of mucinous histology

In multivariate Cox regression analysis, mucinous histology was a strong predictor of OS in the CAIRO study (HR 1.80; 95%CI 1.24-2.62; $p=0.003$). Other independent negative predictors for OS in metastatic CRC patients were male gender (HR 1.32; 95%CI 1.05-1.64; $p=0.01$), primary tumor localization in the colon (HR 1.55; 95%CI 1.19-2.01; $p=0.01$), WHO performance status 2 (HR 1.92; 95%CI 1.16-3.23; $p=0.01$), abnormal serum LDH (HR 1.85; 95%CI 1.48-2.31;

Table 3. Efficacy of the CAIRO study treatment (capecitabine, oxaliplatin and irinotecan) in patients with MC and AC

		MC n = 50	AC n = 435	p-Value
Median OS	Months (95% CI)	13.2 (10.2-17.9)	19.2 (17.9-20.6)	0.03
Median PFS1	Months (95% CI)	5.3 (3.9-6.6)	7.2 (6.6-8.1)	0.09
Overall response rate 1	CR+PR	5/43 (12%)	144/394 (37%)	0.0006
Disease control rate 1	CR+PR+SD	34/4 (79%)	341/394 (87%)	0.17
Median cycle number	Median (range)	6 (1-42)	12 (0-55)	0.004
Overall toxicity	Grade >3	36 (72%)	287 (66%)	0.43

Abbreviations: OS = overall survival; PFS = progression free survival;
CR = complete response; PR = partial response; SD = stable disease;
MC = mucinous adenocarcinoma; AC = adenocarcinoma

Table 4. Efficacy of the CAIRO2 study treatment (capecitabine, oxaliplatin, bevacizumab with or without cetuximab) in patients with MC and AC

		MC n = 49	AC n = 476	p-Value
Median OS	Months (95% CI)	13.1 (9.9-20.2)	21.5 (19.8-22.9)	0.009
Median PFS	Months (95% CI)	7.2 (5.7-8.6)	10.6 (10.1-11.4)	<0.0001
Overall response rate	CR+PR	4/41 (10%)	222/411 (54%)	<0.0001
Disease control rate	CR+PR+SD	35/41 (85%)	387/411 (94%)	0.03
Median cycle number	Median (range)	6 (1-36)	9 (0-48)	0.006
Overall toxicity	Grade >3	35 (71%)	376 (79%)	0.27

Abbreviations: OS = overall survival; PFS = progression free survival;
CR = complete response; PR = partial response; SD = stable disease;
MC = mucinous adenocarcinoma; AC = adenocarcinoma

$p < 0.0001$), more than 2 metastatic sites involved (HR 2.53; 95%CI 1.89-3.38; $p < 0.0001$), T4 classification (HR 1.59; 95%CI 0.99-2.56; $p = 0.04$), and a poor differentiation grade (HR 1.53; 95%CI 0.89-2.64; $p = 0.006$).

In the CAIRO2 study, mucinous histology remained also strongly associated with OS in multivariate analysis (HR 1.76; 95%CI 1.16-2.67; $p = 0.008$). Other clinicopathological features associated with worse survival in this multivariate model were; WHO performance status 1 (HR 1.36; 95%CI 1.06-1.75; $p = 0.02$), abnormal serum LDH (HR 1.65; 95%CI 1.28-2.12; $p = 0.0001$), N2 classification (HR 1.45; 95%CI 1.05-2.01; $p = 0.05$), and *BRAF* mutations (HR 2.61; 95%CI 1.57-4.32; $p = 0.0002$).

The test for heterogeneity ($p = 0.93$) showed that the adjusted hazard ratios for MC versus AC were similar in the CAIRO (1.80; 95%CI 1.24-2.62) and CAIRO2 study (1.76; 95%CI 1.16-2.67). Therefore these hazard ratios were combined in a meta-analysis which resulted in an overall hazard ratio of 1.78 (95%CI 1.35-2.35) for mucinous histology in advanced CRC patients treated with systemic therapy (Figure 1).

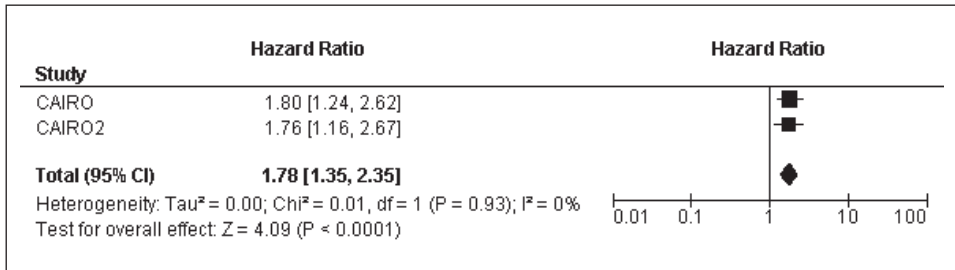


Figure 1. Meta-analysis of both studies estimating the prognostic value of mucinous histology in metastatic CRC treated with systemic therapy.

DISCUSSION

This is the largest retrospective analysis in mucinous metastatic CRC patients treated with the current standard systemic treatments. We demonstrated that MC are less responsive to fluoropyrimidine-based chemotherapy and targeted agents compared to AC. Furthermore, in a multivariate analysis mucinous histology was shown to be a strong negative prognostic factor for survival.

It is recognized that MC are a different entity in patients with CRC. Specific clinical and pathological features are associated with mucinous histology, of which a larger diameter, higher T classification, and extrahepatic localization of metastases were confirmed in our analysis. Patients with MC treated in the CAIRO studies were older compared to the patients with AC, which has not been previously described. In stage II and III CRC, patients with MC were

significantly younger. Mucinous histology is associated with Lynch syndrome²⁷, however, the incidence of MSI in metastatic CRC is low.²³ Therefore, it is likely that the association between mucinous histology and age depends on the presence and cause of a dMMR system.

Our data confirm previous results on differences in molecular signatures between MC and AC in respect to MMR^{10, 28, 29} and *BRAF* mutation status^{14, 30}, supporting the hypothesis that MC are a distinct biologic entity. In previous studies, the incidence of *KRAS* mutations between MC and AC showed opposite results.^{30, 31} However, these data were derived from a small subset of patients and could not be confirmed in our larger patient cohort. Further identification of specific genetic changes in the mucinous phenotype, i.e. using gene expression profiling, are necessary and useful to understand the molecular etiology of these tumors.

Fluoropyrimidines, irinotecan, and oxaliplatin are the effective chemotherapeutic agents used in metastatic CRC. Negri et al.⁶ first reported about the reduced response to 5-FU based chemotherapy of metastatic mucinous CRC. These results were confirmed in a similar subset of patients that received irinotecan and oxaliplatin in addition to fluoropyrimidines as first-line chemotherapy.⁷ Our analysis also showed a highly significant worse response to these chemotherapeutic agents for MC compared to AC. Bevacizumab, a humanized monoclonal antibody against vascular endothelial growth factor (VEGF), combined with 5-FU based chemotherapy is nowadays the standard first-line treatment for metastatic CRC. Our study is the first reporting the outcome of chemotherapy plus targeted agents in MC versus AC. Since the CAIRO2 study¹⁹ had a negative outcome, possibly due to a negative interaction between the study drugs³², the responsiveness of metastatic mucinous CRC to cetuximab cannot be assessed in our series.

The mechanisms for the difference in treatment sensitivity of MC compared to AC remains unclear, but there are several possible explanations. First, differences in molecular features may partly explain the unresponsiveness of tumors with mucinous histology. dMMR is more frequently observed in MC compared to AC, and associated with a reduced response to adjuvant 5-FU treatment in stage II and III CRC patients.^{33, 34} However, the predictive value of dMMR in response to systemic therapy in metastatic CRC is difficult to assess due to its low incidence in metastatic CRC patients.²³ Other molecular markers, such as thymidylate synthase (*TYMS*) and *GSTP1*, are also overexpressed in mucinous CRC³⁵ and possibly correlated with resistance to chemotherapy. We did not investigate these or other chemotherapy pathway markers in our analysis, because they have not been validated for clinical use in metastatic CRC.³⁶ No predictive markers for response to bevacizumab have yet been identified.

Secondly, the mucins themselves may play a role in the ability of tumor cells to escape the effect of systemic therapy. It has been established that mucins play a role in the processes of tumor progression, invasion, survival, and protection against the host immune response.³⁷ MUC2 is a colonic mucin that is overexpressed in the mucinous subtype of CRC³⁸ and correlated with resistance to 5-FU *in vitro*.³⁹ The mucin lakes may also be a physical barrier for the delivery of targeted therapy to the tumors cells, however this has not been investigated.

A third explanation could be that the evaluation of the response to treatment is inadequate in MC patients. In the CAIRO studies we used CT scanning and evaluated response according to RECIST criteria. If neoplastic cells in MC respond to systemic therapy, the total tumor volume is probably more affected by the unresponsive mucin lakes, which could result in false negative conclusions. Our observation that the difference in disease control rate between MC and AC was less compared to the difference in objective response rate supports this hypothesis. If confirmed, the RECIST criteria may not be the optimal instrument to evaluate the objective response rate to treatment in the mucinous subtype of CRC.

The prognostic value of MC is highly controversial, which may be attributed to a large amount of heterogeneous studies with small subsets of patients. Most studies suggested that MC are associated with poor prognosis^{6, 7, 15}, while others found no correlation between histological subtype and clinical outcome.^{2, 16} Our study differs from the published literature in one important aspect, in that only patients with a previous resection of the primary tumor were included, for the obvious reason of tissue availability. Our group has shown that patients with a resection of the primary tumor may have a better prognosis compared to patients without resection.⁴⁰ Since the prognostic value of resection of the primary tumor in metastatic CRC patients has not been clearly established, the possible influence of this parameter cannot be assessed. Therefore we assessed the prognostic value of mucinous histology in metastatic patients with a resection of the primary tumor, and we observed a significant shorter OS in patients with MC compared to AC. As mentioned before, MC present with distinct clinicopathological features, and most of these features are correlated with a worse prognosis. For example, *BRAF* mutations were more frequently observed in MC compared to AC, which is a strong negative prognostic marker in metastatic CRC.¹⁷ However, even with *BRAF* mutations included in a multivariate analysis, mucinous histology remained a strong independent negative prognostic factor. The hazard ratio for mucinous histology is equal or even higher compared to well known stratification factors, such as serum LDH, WHO performance status and number of metastatic sites involved. Appropriate stratification facilitates the interpretation of study results and prevent heterogeneity in response and survival rates. Our findings suggest that mucinous histology should be one of the stratification factors in metastatic CRC trials. Due to the considerable inconsistency in reporting clinicopathological features and use of stratification factors, we believe there is an urgent need for re-establishing the most important prognostic factors in metastatic CRC.

In conclusion, MC are a distinct entity of CRC with specific clinicopathological and genetic features. Mucinous histology is an independent negative prognostic factor for OS in 1010 metastatic CRC patients treated with chemotherapy and targeted agents, which may be explained by resistance to this systemic treatment. We recommend including mucinous histology as a prognostic factor for patients with metastatic CRC.

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Chapter 4 Clinicopathological features and outcome in advanced colorectal cancer patients with synchronous vs metachronous metastases

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ABSTRACT

BACKGROUND: Synchronous metastases of colorectal cancer (CRC) are considered to be of worse prognostic value compared with metachronous metastases, but only few and conflicting data have been reported on this issue.

METHODS: We retrospectively investigated patient demographics, primary tumor characteristics, and overall survival (OS) in 550 advanced CRC patients with metachronous vs synchronous metastases, who participated in the phase III CAIRO study. For this purpose only patients with a prior resection of the primary tumor were considered.

RESULTS: The clinical and pathological characteristics associated with poor prognosis that we observed more often in patients with synchronous metastases (n=280) concerned an abnormal serum lactate dehydrogenase (LDH) concentration ($P=0.01$), a worse WHO performance status ($P=0.02$), primary tumor localization in the colon ($P=0.002$), and a higher T stage ($P=0.0006$). No significant difference in median OS was observed between patients with synchronous metastases and metachronous metastases (17.6 vs 18.5 months, respectively, $P=0.24$).

CONCLUSION: Despite unfavorable clinicopathological features in patients with synchronous metastases with a resected primary tumor compared to patients with metachronous metastases, no difference in the median OS was observed. Possible explanations include a (partial) chemoresistance in patients with metachronous disease because of previous adjuvant treatment, whereas differences between the two groups in screening procedures resulting in a lead time bias to diagnosis or in prognostic molecular markers remain speculative.

INTRODUCTION

Approximately 20% of colorectal cancer (CRC) patients present with synchronous distant metastases at the initial diagnosis, and about 50% of the patients without metastases at presentation develop distant metastases within 3 years of diagnosis.¹ For patients with unresectable metastatic CRC there are no curative options, but a significant benefit in median overall survival can be achieved with palliative systemic treatment.² This treatment consists of cytotoxic chemotherapy (fluoropyrimidines, oxaliplatin, irinotecan) and targeted therapy (VEGF- and EGFR antibodies).

Only few data have been reported on the prognostic role of synchronous and metachronous metastases in patients with advanced CRC treated with chemotherapy, and the results are conflicting. Moreover, there is no consensus about the definition of synchronous and metachronous disease. Synchronous metastases were defined as metastases detected by pre-operative screening or during resection of the primary tumor³⁻⁵, and occurring within three⁶, six^{7,8} or twelve months^{9,10} of the initial diagnosis of CRC. It is not clear whether patients with synchronous vs metachronous metastases may represent two different categories of CRC. Only in some surgical intervention trials the clinicopathological features have been compared between patients with metachronous and synchronous metastases.^{6,7,10} However, these studies involved small numbers of patients, and only limited clinical and pathological features were evaluated.

In a review of 143 phase II and III studies with 21.214 metastatic CRC patients, metachronous vs synchronous metastases were reported as baseline characteristics in only 18 studies.¹¹ Consequently, few data are available on the prognostic value of this parameter with conflicting results.¹²⁻¹⁷

To our knowledge, this is the first large retrospective analysis on the clinical and pathological characteristics of advanced CRC patients with metachronous vs synchronous metastases, and their correlation with outcome. Data were obtained from the phase III CAIRO study of the Dutch Colorectal Cancer Group (DCCG).¹⁸

MATERIALS AND METHODS

Patients

Data were used from the phase III CAIRO study of the DCCG.^{18, 19} In this study patients were randomized between sequential and combination treatment with capecitabine, irinotecan, and oxaliplatin. Stratification parameters included WHO performance status, serum lactate dehydrogenase (LDH), prior adjuvant therapy, predominant localization of metastases, and participation institution. Assessment of tumor response was scheduled every three cycles (nine weeks) according to RECIST criteria.²⁰ Follow-up after completion of treatment was performed every 3 months until death. The primary endpoint was overall survival (OS).

Patients were divided into synchronous and metachronous disease, with synchronous disease defined as distant metastases occurring within, and metachronous disease beyond 6 months of the primary diagnosis of CRC. For two reasons only patients in whom a resection of the primary tumor had been performed were included in the analysis. First, tissue of the primary tumor was required for histopathological review. Second, the arguments for non-resection may greatly vary, from patients with an asymptomatic primary and excellent performance status to patients with a symptomatic primary with extensive metastases and poor performance status in whom a delay in systemic treatment is not warranted. These arguments are often not recorded in the patients' files.

Pathologic procedures and parameters

Standardized pathology examination was performed in the pathology laboratories of the referring hospitals. The maximum diameter was noted with an invasion depth described in terms of the T classification and lymph node stage in terms of the N classification (TNM).²¹ All reports of these examinations with haematoxylin and eosin stained coupes of the primary tumors were collected. Histopathologic review was carried out by two independent observers. If the scoring was not unambiguous, the two observers discussed until agreement was reached. Classification of the tumors was performed using the World Health Organization guidelines.²² A tumor was considered to be of the mucinous type when at least 50% of the tumor was mucinous. The tumors were graded according to the grade of differentiation into well, moderately and poorly adenocarcinomas on the basis of the part of poorest differentiation in the tumor. Growth pattern, the presence of inflammatory reaction and fibroblastic reaction were assessed according to Jass.²³ The mismatch repair system (MMR) status was determined by immunohistochemistry and microsatellite instability (MSI) analysis.²⁴

Statistical analysis

The comparison of patient and primary tumor characteristics between patients with synchronous and metachronous metastases was done using Wilcoxon's rank sum test or χ^2 -test where appropriate. The progression free survival (PFS) for first line treatment was calculated from the date of randomization to the first observation of disease progression or death from any cause. OS and PFS curves were estimated using the Kaplan-Meier method and compared with

Table 1. Baseline characteristics

		Metachronous (n=270)	Synchronous (n=280)	p-Value
Age at randomization	Median (range)	66.0 (31.0-79.0)	62.5 (34.0-81.0)	<0.0001 ^a
Gender	Male	163 (60%)	179 (64%)	0.43 ^b
	Female	107 (40%)	101 (36%)	
Predominant localization metastases	Liver	131 (49%)	240 (86%)	<0.0001 ^b
	Extrahepatic	135 (50%)	39 (14%)	
	Unknown	4 (1%)	1 (<1%)	
WHO performance status at randomization	0 and 1	265 (98%)	262 (94%)	0.02 ^b
	2	5 (2%)	17 (6%)	
	Unknown		1 (<1%)	
Serum LDH at randomization	Normal	183 (68%)	161 (58%)	0.01 ^b
	> ULN	84 (31%)	117 (42%)	
	Unknown	3 (1%)	2 (<1%)	
Site of primary tumor	Colon	114 (42%)	149 (53%)	0.002 ^b
	Rectosigmoid	65 (24%)	73 (26%)	
	Rectum	91 (34%)	58 (21%)	
Prior adjuvant therapy	No	189 (70%)	276 (99%)	<0.0001 ^b
	Yes	81 (30%)	3 (1%)	
	Unknown		1 (<1%)	
Metastatic sites involved	1	121 (45%)	141 (50%)	0.63 ^b
	2	96 (35%)	95 (34%)	
	>2	45 (17%)	44 (16%)	
	Unknown	8 (3%)		
Treatment arm	Sequential	130 (48%)	138 (49%)	0.80 ^b
	Combination	140 (52%)	142 (51%)	

Abbreviations: ULN = upper limit of normal; WHO = World Health Organization

^aWilcoxon's rank sum test; ^b χ^2

Table 2. Primary tumor characteristics

		Metachronous (n = 270)	Synchronous (n = 280)	p-Value
Diameter	Median (range)	40.0 (15.0-120.0)	62.5 (34.0-81.0)	0.007 ^a
Invasion depth	T 1-2	28 (10%)	7 (3%)	0.0006 ^b
	T 3	187 (69%)	200 (71%)	
	T 4	45 (17%)	59 (21%)	
	Unknown	10 (4%)	14 (5%)	
Lymph node status	N 0	104 (39%)	50 (18%)	<0.0001 ^b
	N 1	97 (36%)	97 (35%)	
	N2	56 (21%)	113 (40%)	
	Unknown	13 (5%)	20 (7%)	
Classification	Adenocarcinoma	216 (80%)	217 (78%)	0.53 ^b
	Adenocarcinoma with mucinous component	28 (10%)	33 (12%)	
	Mucinous carcinoma	24 (9%)	24 (8%)	
	Other	2 (1%)	6 (2%)	
Differentiation grade	Well	11 (4%)	11 (4%)	0.15 ^b
	Moderate	143 (53%)	125 (45%)	
	Poor	115 (43%)	141 (50%)	
	Unknown	1 (<1%)	3 (1%)	
Infiltration pattern	Circumscribed	69 (26%)	48 (17%)	0.02 ^b
	Diffuse	199 (74%)	226 (81%)	
	Unknown	2 (<1%)	6 (2%)	
Fibroblastic reaction	None/little	84 (31%)	76 (27%)	0.39 ^b
	Extensive	184 (68%)	196 (70%)	
	Unknown	2 (1%)	8 (3%)	
Lymphoid reaction	None/little	193 (71%)	218 (78%)	0.04 ^b
	Extensive	74 (27%)	55 (20%)	
	Unknown	3 (1%)	7 (2%)	
MSI status	pMMR	261 (97%)	271 (97%)	0.94 ^b
	dMMR	9 (3%)	9 (3%)	

Abbreviations: MSI = microsatellite instability; pMMR = proficient mismatch repair system; dMMR = deficient mismatch repair system

^aWilcoxon's rank sum test; ^b χ^2

the log-rank test. Multivariate analysis of survival was performed by means of a Cox proportional hazard model. Patients were considered evaluable for response if they had completed at least 3 cycles of chemotherapy. Overall response was defined as partial response or complete response. Disease control was defined by stable disease with a duration of more than 4 months or partial response or complete response.²⁰ Differences in response and disease control rates were analysed by a χ^2 (univariate) model. All tests were two-sided and P-values of less than 0.05 were considered statistically significant. The analyses were performed using SAS 8.2 software.

RESULTS

Patient characteristics

In 550 of 803 eligible patients in the CAIRO study, a resection of the primary tumor was performed and material for histological review of the primary tumor was available, and these 550 patients were included in this analysis. Compared to the metachronous group (n=270), patients with synchronous metastases (n=280) were younger ($p<0.0001$), had more often an abnormal serum LDH at randomization ($p=0.01$), and more often the liver as predominant site of metastases ($p<0.0001$). Primary tumor localization in the colon ($p=0.002$), a worse WHO performance status at randomization ($p=0.02$), and no previous adjuvant chemotherapy ($p<0.0001$) were more frequently observed in patients with synchronous metastases (Table 1).

Primary tumor characteristics

Tumors of patients with synchronous metastases had larger diameters ($p=0.007$), a higher T ($p=0.0006$) and N stage ($p<0.0001$), absent or little lymphoid reaction ($p=0.04$) and more frequently a diffuse infiltration pattern ($p=0.02$) than patients with metachronous disease. There were no significant differences between the synchronous and metachronous group in terms of classification, differentiation grade, MSI status, and fibroblastic reaction surrounding the tumor (Table 2).

Correlation of clinical and pathological characteristics with outcome

The effect of clinical and pathological characteristics on median OS was evaluated. In the overall population of 550 patients, the following parameters significantly correlated with the median OS: predominant liver localization of metastases yes vs no (17.9 vs 19.5 months,

respectively; $p=0.02$), WHO performance status 2 vs 0-1 (6.2 vs 18.5 months, respectively; $p<0.0001$), serum LDH concentration abnormal vs normal (12.8 vs 21.3 months, respectively; $p<0.0001$), and number of metastatic sites involved >2 vs 2 vs 1 (12.4 vs 18.0 vs 21.4 months, respectively; $p<0.0001$). In the effect on median OS a significant trend was observed for the following pathological characteristics of the primary tumor: T4 vs T3 vs T1-2 (14.3 vs 18.9 vs 21.9 months, respectively; $p=0.03$), N2 vs N1 vs N0 (14.4 vs 18.9 vs 20.7 months, respec-

Table 3. Prognostic value of clinical and pathological characteristics for OS (multivariate analysis)

Multivariate analysis for OS		Hazard ratio (95%CI)	p-Value
Onset of metastasis	Metachronous	1.05 (0.81-1.36)	0.74
	Synchronous	R	
Gender	Female	0.78 (0.63-0.97)	0.03
	Male	R	
Site of primary tumor	Colon	1.29 (0.98-1.70)	0.0008
	Rectosigmoid	0.78 (0.58-1.04)	
	Rectum	R	
WHO performance status at randomization	0 and 1	0.53 (0.32-0.88)	0.01
	2	R	
Serum LDH at randomization	$> \text{ULN}$	1.79 (1.44-2.23)	<0.0001
	normal	R	
Number of metastatic sites involved	1	0.40 (0.30-0.53)	<0.0001
	2	0.55 (0.41-0.75)	
	>2	R	
Invasion depth	T 1-2	0.69 (0.42-1.12)	0.04
	T 3	0.72 (0.55-0.93)	
	T4	R	
Classification	Adenocarcinoma	1.00 (0.44-2.25)	0.007
	Adenocarcinoma with mucinous component	1.56 (0.66-3.68)	
	Mucinous carcinoma	1.71 (0.71-4.13)	
	Other	R	
Differentiation grade	Well	0.69 (0.40-1.20)	0.01
	Moderate	0.73 (0.59-0.91)	
	Poor	R	

Abbreviations: R = reference group; ULN = upper limit of normal; WHO = World Health Organization

tively; $p=0.003$), mucinous adenocarcinoma vs adenocarcinoma with mucinous component vs adenocarcinoma (13.5 vs 13.7 vs 19.3 months, respectively; $p=0.006$), and differentiation grade poor vs moderate vs well (14.8 vs 20.4 vs 24.9 months, respectively; $p=0.0001$). By univariate analysis no effect on median OS was found for age, gender, site of the primary tumor, prior adjuvant therapy, treatment arm, infiltration pattern, fibroblastic reaction, lymphoid reaction, and MSI status.

In a multivariate model all patient and primary tumor characteristics were included. Independent predictors for median OS in advanced CRC patients, were T stage ($p=0.04$), differentiation grade ($p=0.01$), classification ($p=0.007$), serum LDH at randomization ($p<0.0001$), WHO performance status ($p=0.01$), site of the primary tumor ($p=0.0008$), gender ($p=0.03$), and metastatic sites involved ($p<0.0001$) (Table 3).

Outcome in patients with metachronous vs synchronous metastases

No significant difference in median OS was observed for patients with metachronous vs synchronous metastases in an univariate analysis (18.5 vs 17.6 months, respectively; $p=0.24$) (Figure 1). In addition, to assess a possible effect of tumor burden, we compared the largest diameter of liver metastases between the two groups, and no difference was observed ($p>0.05$, data not shown). In a multivariate model, in which all patient and primary tumor characteristics were included, the hazard ratio for metachronous vs synchronous metastases was 1.05 (95% CI 0.81-1.36; $p=0.74$) (Table 3).

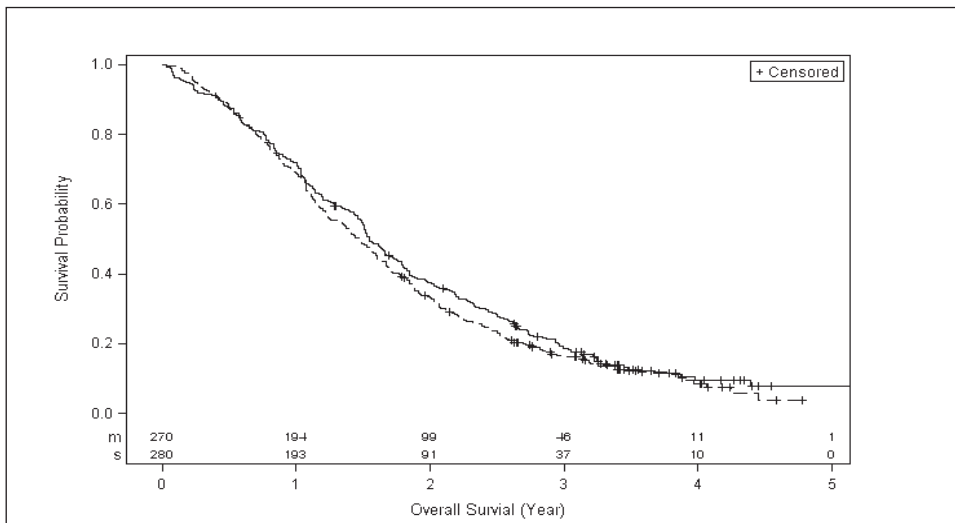


Figure 1. Kaplan-Meier curve for overall survival of advanced CRC patients with metachronous (---) and synchronous (—) metastases in whom a resection of the primary tumor was performed.

The median PFS in first line treatment was not significantly different between patients with metachronous vs synchronous metastases (7.2 vs 6.6 months, respectively; $p=0.23$). Overall, 494 patients were assessable for response in first line treatment: 235 in the metachronous group and 259 in the synchronous group. The overall response rate (complete plus partial tumor response) in first line treatment was significantly better in patients with synchronous metastases compared to patients with metachronous metastases (38% vs 28%, respectively; $p=0.02$). The disease control rate (complete plus partial tumor response plus stable disease) in first line treatment was not significantly different between patients with synchronous and metachronous metastases (81% vs 87%, respectively; $p=0.11$).

Interaction of worse prognostic factors in patients with synchronous vs metachronous metastases

Patients with synchronous vs metachronous metastases in whom a resection of the primary tumor was performed showed significantly different clinical and pathological characteristics. Most of these clinicopathological features were correlated with outcome in the total study population. However, despite the presence of factors associated with poor prognosis, patients with synchronous metastases had no worse survival compared to patients with metachronous metastases.

To find a possible explanation for this observation we analysed whether the median OS of patients with individual clinical and pathological characteristics was significantly different between the synchronous and metachronous group. However, this proved not to be the case ($p>0.05$ for all analyses).

Next, we compared the number of worse prognostic factors *per patient* between the synchronous and metachronous group to detect whether there was a skewed distribution. Again, this analysis showed no significant difference in the distribution of these characteristics per patient between the synchronous and metachronous group ($p>0.05$ for all analyses).

DISCUSSION

In this retrospective analysis of the phase III CAIRO trial, we observed that CRC patients with synchronous metastases, in whom the primary tumor was resected, significantly more often had clinical and pathological characteristics associated with poor prognosis compared to patients with metachronous metastases.

There is no consensus in the literature on the definition of synchronous vs metachronous metastases. We selected a cut-off value of 6 months after the initial diagnosis for two reasons. First, in some patients a staging procedure is performed only after full recovery from surgery of the primary tumor, which may take several months in some patients. A 6-month period will assure adequate classification of these patients. Second, metastases developing during the first 6 months after surgery of the primary tumor probably reflect similar tumor biology compared with metastases detected at initial diagnosis. Therefore, we consider a 6-month cut-off value to be a clinically useful distinction between synchronous and metachronous disease.

The unfavorable clinical characteristics that we observed more often in patients with synchronous disease concerned a worse performance status, an abnormal serum LDH, and the colon as the primary site of the tumor. Only the primary site of the tumor has been previously described as being different between synchronous and metachronous disease.^{6, 10} We identified a higher T stage of the primary tumor as an independent worse prognostic factor for median OS, which we observed more in patients with synchronous metastases. This confirms previously reported results of smaller series.^{5, 7, 10}

Despite these poor baseline characteristics in patients with synchronous metastases, the median OS was not decreased compared to patients with metachronous metastases. Tsai et al.¹⁰ found differences in diameter, number, and distribution of liver metastases between patients with synchronous and metachronous disease, and concluded that these characteristics were of significant importance for survival. Tumor burden, as determined by the largest diameter of measurable disease and the number of metastatic sites, were comparable between patients with synchronous and metachronous metastases, indicating that this parameter did not influence our results. However, several other factors may explain this unexpected finding. First, a significant percentage of patients with metachronous metastases were treated with prior adjuvant chemotherapy, whereas patients with synchronous metastases obviously were not. Theoretically, this may have resulted in a (partial) resistance to chemotherapy in the former group. Indeed, we observed a higher overall response rate to first line chemotherapy in patients with synchronous metastases, suggesting that this may compensate the presence of worse prognostic factors in this group. Second, there may be heterogeneity between and also within the groups of patients with synchronous and metachronous disease with regard to symptomatic vs asymptomatic disease and, in the latter situation, a lead time bias caused by different time schedules for screening. Third, survival of CRC patients could be influenced by a difference in presence of prognostic molecular markers between patients with synchronous vs metachronous metastases.⁸

Comparing our results with the literature, only few chemotherapy trials performed proportional hazard models to determine the influence of metachronous and synchronous disease on median OS. Several authors showed no prognostic role for these parameters^{13, 14, 17, 25, 26} whereas others identified metachronous disease as a favorable prognostic parameter.^{12, 15, 16, 27}

Our analysis differs from the published literature in one important aspect, in that only patients with a previous resection of the primary tumor were included in the synchronous group. If patients with both resected and non-resected primary tumors were included in the synchronous group, a significant median OS benefit was observed for patients with metachronous vs synchronous metastases.²⁸ Therefore, the conflicting results of previous studies on the prognostic role of synchronous disease may be caused by differences among these studies in the status of the resection of the primary tumor. Support for our data is provided by two recent prospective analyses in which no difference in OS was observed between patients with resected synchronous vs resected metachronous CRC liver and lung metastases, with a resection of the primary tumor having been performed in all patients.^{5, 6}

In conclusion, despite the presence of factors associated with poor prognosis in patients with synchronous metastases, the parameter of synchronous and metachronous metastases was not of prognostic value in advanced CRC patients in whom a resection of the primary tumor was performed. Possible explanations include a (partial) chemoresistance in patients with metachronous disease because of prior adjuvant treatment, whereas differences between the two groups in screening procedures resulting in a lead time bias to diagnosis or in prognostic molecular markers remain speculative.

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Chapter 5 ***KRAS* mutation analysis: a comparison between primary tumors and matched liver metastases in 305 colorectal cancer patients**

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ABSTRACT

BACKGROUND: *KRAS* mutation is a negative predictive factor for treatment with anti-epidermal growth factor receptor (EGFR) antibody in metastatic colorectal cancer (CRC). *KRAS* mutation analysis is usually performed on primary tumor tissue because metastatic tissue is often not available. However, controversial data are available on the concordance of test results between primary tumors and corresponding metastases. We assessed the concordance of *KRAS* mutation status in a study of 305 primary colorectal tumors and their corresponding liver metastases.

METHODS: Patients with histologically confirmed CRC who underwent surgical resection of the primary tumor and biopsy or surgical resection of the corresponding liver metastasis were included. *KRAS* mutation analysis was performed for codons 12 and 13.

RESULTS: *KRAS* mutation was detected in 108 out of 305 primary tumors (35.4%). In 11 cases (3.6%) we found a discordance between primary tumor and metastasis: 5 primary tumors had a *KRAS* mutation with a wild-type metastasis, 1 primary tumor was wild-type with a *KRAS* mutation in the metastasis, and in 5 cases the primary tumor and the metastasis had a different *KRAS* mutation.

CONCLUSION: We observed a high concordance of *KRAS* mutation status of 96.4% (95% CI 93.6-98.2%) between primary colorectal tumors and their corresponding liver metastases. In only 6 patients (2.0%; 95% CI 0.7-4.2%) the discordance was clinically relevant. In this largest and most homogenous study to date, we conclude that both primary tumors and liver metastases can be used for *KRAS* mutation analysis.

INTRODUCTION

Recent advances in specific signalling pathways of cancer cells have introduced targeted therapy into treatment regimes for patients with metastatic colorectal cancer (CRC).¹ Cetuximab and panitumumab are monoclonal antibodies that bind to the extracellular domain of the epidermal growth factor receptor (EGFR). They inhibit ligand-induced stimulation of several intracellular signalling pathways, such as RAS/RAF/MAPK and phosphoinositide-3 pathway, which results in decreased stimulation of cell cycle progression, proliferation, angiogenesis, and stimulation of apoptosis.² The *KRAS* oncogene is currently the most relevant molecular biomarker that predicts the response to EGFR-targeted therapy in CRC. An oncogenic mutation in *KRAS* leads to constitutive activation of the RAS/RAF signalling pathway independent from EGFR activation by binding of the ligand.³ *KRAS* mutations occur in approximately 38% of colorectal tumors and involve codon 12 and 13 in more than 96% of cases.⁴ Metastatic CRC patients with tumors harboring a *KRAS* mutation are resistant to treatment with anti-EGFR antibodies, showing lower response rates, decreased progression free survival, and overall survival compared to patients with *KRAS* wild-type tumors.⁵⁻⁷ Therefore, the European Medicines Agency and the Food and Drug Administration have restricted the use of anti-EGFR antibodies in metastatic CRC to patients with *KRAS* wild-type tumors.

Cetuximab and panitumumab have shown efficacy both as monotherapy^{6, 8} and in combination with chemotherapy^{5, 7} in patients with *KRAS* wild-type metastatic CRC. Nevertheless, even among patients with *KRAS* wild-type tumors, the majority of patients does not respond to anti-EGFR therapy. Efficacy of anti-EGFR therapy was suggested to be further restricted to patients with *BRAF* wild-type tumors.⁹ An additional explanation for the suboptimal response rates to anti-EGFR antibodies in patients with *KRAS* wild-type tumors is discordance of *KRAS* mutation status between primary colorectal tumors and corresponding metastases. In the early dissemination model, tumor cells depart the primary lesion before the acquisition of a fully malignant phenotype to undergo new mutations and metastatic growth at a distant site.¹⁰ According to this model, a discordance in mutation status between primary tumors and metastases may occur, and as a consequence the mutation status of the primary tumor might not be adequate to predict the response of metastases to anti-EGFR treatment.

Current data on the concordance in *KRAS* mutation status between primary colorectal tumors and metastases are conflicting. Five studies showed a 100% concordance of *KRAS* mutation status in primary CRC and corresponding metastases.¹¹⁻¹⁵ In contrast to these data, others have reported a discordance of *KRAS* mutation status in primary tumors and metastatic sites, with an overall discordance observed in 4-32% of the patients.¹⁶⁻²⁸ These controversial results are probably due to the fact that these studies were underpowered with a small number of patients, and included a wide variety of metastatic sites. Therefore it is still uncertain whether the

evaluation of *KRAS* mutation status in the most commonly available primary tumor correctly reflects the *KRAS* mutation status of corresponding metastasis. This is highly relevant given the large number of CRC patients as well as the potential toxicity and costs of anti-EGFR therapy. We assessed the concordance in *KRAS* mutation status in primary tumors and their corresponding liver metastases in an adequately powered study of 305 CRC patients.

PATIENTS AND METHODS

Patient selection

Patients with histologically confirmed CRC who underwent surgical resection of the primary tumor and biopsy or surgical resection of the corresponding liver metastasis were included in this analysis. Results were obtained from archived material of three large pathology laboratories and from material collected from the CAIRO2 study, a large multicentre trial of the Dutch Colorectal Cancer Group (DCCG).⁵

In patients with a discordance of *KRAS* mutation status between the primary tumor and metastasis, additional blocks of the primary tumor were obtained to exclude heterogeneity within the tumor. Lymph node metastases present at the time of diagnosis were also acquired in these patients.

Tumor DNA preparation

Formalin-fixed paraffin-embedded tissue blocks were cut at 4 μm thickness and stained with heamatoxylin and eosin (HE). The presence of tumor tissue was marked by a pathologist. Subsequently the blocks were cut at 20-40 μm thickness and micro dissected for DNA extraction. Tumor tissue was dissolved in 200 μl lysis buffer (QIAamp DNA Micro Kit, Qiagen, The Netherlands) and incubated with proteinase K overnight at 56°C for two nights. DNA was extracted according to the manufacturer's protocol (QIAamp DNA Micro Kit, Qiagen, The Netherlands), and DNA concentration was determined at 260 nm using the Nanodrop 26 ND-1000 spectrophotometer (Nanodrop Technologies Inc, Wilmington, USA).

KRAS mutation analysis

For *KRAS* mutation analysis, exon 2 (codon 12 and 13) was amplified using a 50 μl reaction mixture containing 0.2 μM forward (TGTAACACGACGGCCAGTAGGCCTGCTGAAAATGACTG) and reverse (CAGGAAACAGCTATGACCTGGATCATATTCGTCCACAAAA) primer (Invitrogen,

The Netherlands); dATP, dCTP, dGTP and dTTP (GE Healthcare, The Netherlands) at 0.2 mM each; 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 2.5 mM MgCl₂; 1 U AmpliTaq Gold polymerase (Applied Biosystems, Nieuwkerk a/d IJssel, The Netherlands) and 50 ng of template DNA. The PCR conditions were as follows: 94°C for 10 min; 92°C for 1 min, 60°C for 1 min, 72°C for 1 min (40 cycles); and 72°C for 10 minutes.

All PCR products were purified with the MultiScreen HTS 96 well Filtration System (Millipore, Ireland). Subsequently, the purified products were sequenced using fluorescently labeled terminators (BigDye® Terminators (v 1.1); Applied Biosystems, USA) with both M13-forward and M13-reverse sequencing primers. The sequencing products were analyzed on an ABI 3730 DNA Analyzer (Applied Biosystems) and the data analysis was performed using Sequencing Analysis Software Sequencing Analysis Software v5.3.1 with KBTM Basecaller. Sequence results were scored by visual inspection of the chromatograms.

Statistical analysis

We considered a discordance level of 5% or more to be clinically relevant, that is, leading to substantial change in routine clinical practice. To exclude such level of discordance under the assumption that the true discordance was 2.5% or less, we set the sample size at 304 paired samples. With this sample size, the precision in the estimated percentage of discordance was 2.5% (i.e. standard error 1.25, half-width of the 95%-confidence interval equal to 2.5%).

The comparison of patient and primary tumor characteristics between patients with *KRAS* wild-type and *KRAS* mutant primary tumors was done using Wilcoxon's rank sum test or χ^2 for numerical or categorical variables, respectively. Differences in *KRAS* mutation status between the primary tumor and corresponding metastasis were analyzed by calculating the percentage of concordance, and (clinically relevant) discordance, together with the corresponding Clopper-Pearson 95% confidence intervals. Differences were considered to be statistically significant when the P-value was below 0.05. All statistical tests were two-sided.

RESULTS

Patient characteristics

We analyzed *KRAS* codon 12 and 13 mutations in 320 matched primary colorectal tumors and liver metastases. The tumor cell percentages in all primary tumors and metastases were above 30%. We failed to obtain a *KRAS* mutation status in 15 patients, therefore our further analyses were performed in 305 paired samples. Patient characteristics are shown in Table 1.

Table 1. Distribution of tumor characteristics according to *KRAS* status of the primary tumor

	Overall n = 305	<i>KRAS</i> mutation n=108	<i>KRAS</i> wild-type n=197	p-Value
Age				0.20
Median (IQR)	64 (57-70)	65 (58-71)	64 (57-70)	
Gender				0.37
Male	191 (62.6%)	64 (59.3%)	127 (64.5%)	
Female	114 (37.4%)	44 (40.7%)	70 (35.5%)	
Metastases presentation				0.45
Synchronous	169 (55.4%)	63 (58.3%)	106 (53.8%)	
Metachronous	136 (44.6%)	45 (41.7%)	91 (46.2%)	
Tumor location				0.63
Colon	167 (54.8%)	59 (54.6%)	108 (54.8%)	
Rectum	54 (17.7%)	16 (14.8%)	38 (19.3%)	
Rectosigmoid	80 (26.2%)	32 (29.6%)	48 (24.4%)	
Unknown	4 (1.3%)	1 (0.9%)	3 (1.5%)	
Histopathological subtype				0.12
Adenocarcinoma	271 (88.9%)	90 (83.3%)	181 (91.9%)	
Adenocarcinoma with mucinous component	21 (6.9%)	10 (9.3%)	11 (5.6%)	
Mucinous adenocarcinoma	8 (2.6%)	5 (4.6%)	3 (1.5%)	
Unknown	5 (1.6%)	3 (2.8%)	2 (1.0%)	
Differentiation grade				0.21
Good	33 (10.8%)	13 (12.0%)	20 (10.2%)	
Moderate	196 (64.3%)	65 (60.2%)	131 (66.5%)	
Poor	52 (17.0%)	17 (15.7%)	35 (17.8%)	
Unknown	24 (7.9%)	13 (12.0%)	11 (5.6%)	
T stage				0.62
T1	4 (1.3%)	2 (1.9%)	2 (1.0%)	
T2	20 (6.6%)	9 (8.3%)	11 (5.6%)	
T3	231 (75.7%)	81 (75.0%)	150 (76.1%)	
T4	36 (11.8%)	11 (10.2%)	25 (12.7%)	
Unknown	14 (4.6%)	5 (4.6%)	9 (4.6%)	
N stage				0.10
N0	114 (37.4%)	46 (42.6%)	68 (34.5%)	
N1	87 (28.5%)	31 (28.7%)	56 (28.4%)	
N2	86 (28.2%)	26 (24.1%)	60 (30.5%)	
Unknown	18 (5.9%)	5 (4.6%)	13 (6.6%)	
Number of lymph nodes examined				0.28
Median (IQR)	10 (6-15)	10 (6-13)	10 (6-16)	
Number of lymph node metastases				0.15
Median (IQR)	1 (0-4)	1 (0-3)	1 (0-4)	

Abbreviation: IQR = interquartile range

KRAS mutation and histopathological parameters

A total of 108 patients (35.4%) had a *KRAS* mutation in the primary tumor; of which 37 patients had a Gly12Asp mutation, 28 patients a Gly12Val mutation, 14 patients a Gly13Asp mutation, 10 patients a Gly12Cys mutation, 7 patients a Gly12Ser mutation, 7 patients a Gly12Ala mutation, 3 patients a Gly12Arg mutation, 1 patient a Gly12Asp and Gly12Ala mutation, and 1 patient a Gly12Phe mutation (Table 2). Histopathological characteristics of the primary tumor were comparable between patients with and without a *KRAS* mutation (Table 1).

Concordance of *KRAS* status in primary tumors and corresponding liver metastases

In 294 patients (96.4%; 95% CI 93.6-98.2%), the same *KRAS* mutation status was obtained from the primary tumor and the corresponding liver metastasis. In 11 patients (3.6%; 95% CI 1.8-6.4%), of which 7 had synchronous metastases at diagnosis and 4 developed metachronous

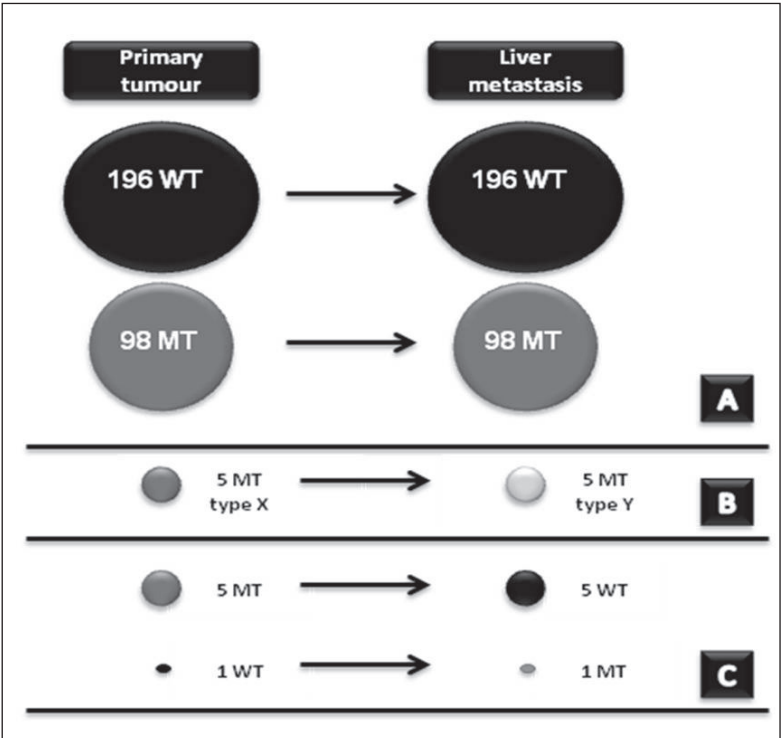


Figure 1. Overall concordance of the *KRAS* mutation status between primary tumor and liver metastasis (A), discordance without clinical impact (B) and discordance with clinical impact (C). Abbreviations: WT = wild-type; MT = mutation

metastases, we found a discordance between primary tumors and metastases. Five patients had a *KRAS* mutation in the primary tumor and not in the liver metastasis. Only one patient had a wild-type status of the primary tumor, while the metastasis showed a *KRAS* mutation. In 5 patients the primary tumors had different *KRAS* mutations compared with the metastases. One of these patients had two primary tumors. Both primary tumors had the same *KRAS* mutation (Gly13Asp), while the liver metastasis had a different *KRAS* mutation (Gly12Ser). In another patient the primary tumor had a double mutation (Gly12Asp/Gly12Val) and the metastasis had a Gly12Asp mutation (Figure 1, Table 3). Taken together, the observed discordance was clinically relevant in only six patients (2.0%; 95% CI 0.7-4.2%).

Subsequent analyses in patients with a discordance of *KRAS* status

Several tests were performed to exclude bias of the test results. First, the HE coupes of all patients with a discordant *KRAS* mutation status between the primary tumor and liver metastasis were revised. The primary tumors and liver metastases had a mean tumor cell percentage of 65 and 60%, respectively. Subsequent independent reanalysis of the *KRAS* mutation status resulted in the same discordances.

Second, several mutation analyses were performed on different areas of the tumor and from different tumor blocks in order to establish possible tumor heterogeneity. Two patients showed heterogeneity of *KRAS* status within the primary tumor. One of these patients demonstrated two areas with a Gly12Asp mutation and one area with wild-type status, of which the latter resembled the liver metastasis. The other patient showed two different *KRAS* mutations within the same tumor, of which one is concordant with the liver metastasis (Table 3).

Third, six of the eleven patients with discordant results did have lymph node metastases at the time of diagnosis. *KRAS* mutation testing of all lymph nodes separately revealed overall

Table 2. Distribution of *KRAS* mutation types

Codon 12 / 13	Patients with <i>KRAS</i> mutation (n, %)
Gly12Asp	37 (34%)
Gly12Val	28 (26%)
Gly13Asp	14 (13%)
Gly12Cys	10 (9%)
Gly12Ser	7 (6%)
Gly12Ala	7 (6%)
Gly12Arg	3 (3%)
Gly12Phe	1 (1%)
Gly12Asp + Gly12Ala	1 (1%)

Table 3. Patients with a discordant *KRAS* status between primary tumor and metastasis

	<i>KRAS</i> status primary tumor	<i>KRAS</i> status 2 nd tumor	<i>KRAS</i> status lymph node metastasis	<i>KRAS</i> status liver metastasis
1	Gly12Ala	-	LN 1: Gly12Ala	WT
			LN 2: Gly12Ala	
			LN 3: Gly12Ala	
2	Gly12Asp	-	-	WT
	Gly12Asp			
	WT			
3	Gly12Cys	-	-	WT
4	Gly12Asp	-	LN 1: Gly12Asp	WT
	Gly12Asp		LN 2: Gly12Asp	
	Gly12Asp		LN 3: Gly12Asp	
	Gly12Asp		LN 4: Gly12Asp	
			LN 5: WT	
5	Gly12Ser	-	-	WT
6	WT	-	-	Gly12Cys
7	Gly12Asp	-	LN 1: WT	Gly12Ala
			LN 2: WT	
			LN 3: WT	
8	Gly13Asp	Gly13Asp	LN 1: Gly13Asp	Gly12Ser
9	Gly12Ser	-	-	Gly12Ala
10	Gly12Cys	-	LN 1: Gly12Asp	Gly12Asp
	Gly12Asp		LN 2: Gly12Asp	
			LN 3: Gly12Asp	
			LN 4: Gly12Asp	
			LN 5: Gly12Asp	
			LN 6: WT	
11	Gly12Asp/ Gly12Val	-	LN 1: Gly12Val	Gly12Asp
			LN 2: Gly12Val	
			LN 3: Gly12Val	
			LN 4: Gly12Asp	
			LN 5: Gly12Asp	
			LN 6: Gly12Asp	
			LN 7: Gly12Asp	

Multiple blocks of primary tumor tissue and lymph node metastases were tested when available

Abbreviation: WT = wild-type

Table 4. Overview of studies providing data on *KRAS* status of primary tumor and related metastases

Author study	Year	No. of pts	Analyzed metastatic site	Method	<i>KRAS</i> mutation in PT (%)	<i>KRAS</i> mutation in PT, WT in M	<i>KRAS</i> WT in PT, mutation in M	Total percentage of discordance
Albanase	2004	30	liver	SSCP analysis	14 (47%)	5/14 (36%)	4/16 (25%)	9/30 (30%)
Al-Mulla	1998	26	liver	ASO/direct seq	10 (38%)	2/10 (20%)	3/16 (19%)	5/26 (19%)
		31	lymph node	ASO/direct seq	10 (32%)	1/10 (10%)	5/21 (24%)	6/31 (19%)
Artale	2008	48	diverse, 81% liver	direct seq	11 (23%)	1/11 (9%)	2/37 (5%)	3/48 (6%)
Baldus	2010	20	visceral metastasis	direct seq	9 (45%)	1/9 (11%)	1/11 (9%)	2/20 (10%)
		55	lymph node	direct seq	29 (53%)	15/29 (52%)	2/26 (8%)	17/55 (31%)
Cejas	2010	93	liver	direct seq	30 (32%)	1/30 (3%)	4/63 (6%)	5/93 (5%)
		17	lung	direct seq	10 (59%)	1/10 (10%)	1/7 (14%)	2/17 (12%)
Etienne-Grimaldi	2008	48	liver biopsy	PCR-RFLP	16 (33%)	0 (0%)	0 (0%)	0 (0%)
Italiano	2009	59	not specified	seq	23 (39%)	1/23 (4%)	2/36 (6%)	3/59 (5%)
Losi	1992	19	local recurrence	multiplex-ASPCR	12 (63%)	0 (0%)	0 (0%)	0 (0%)
		16	metastasis, 38% liver	multiplex-ASPCR	13 (81%)	0 (0%)	0 (0%)	0 (0%)
Loupakis	2009	43	liver	seq	not mentioned	0 (0%)	2/*	2/43 (5%)
Molinari	2009	37	diverse, 74% liver	seq	16 (43%)	2/16 (13%)	1/21 (5%)	3/37 (8%)
		15	lymph node	seq	8 (53%)	0 (0%)	0 (0%)	0 (0%)
Oliveira	2006	28	lymph node	not mentioned	18 (64%)	2/18 (11%)	7/10 (70%)	9/28 (32%)
Oudejans	1991	31	liver and lung	hybridization	14 (45%)	1/14 (7%)	1/17 (6%)	2/31 (6%)
Perrone	2008	10	diverse, mainly liver	direct seq	2 (20%)	1/2 (50%)	1/8 (13%)	2/10 (20%)
Santini	2008	99	diverse, 80% liver	seq	38 (38%)	3/38 (8%)	1/61 (2%)	4/99 (4%)
Garm Spindler	2009	31	not specified	qPCR	11 (35%)	2/11 (18%)	0/20 (0%)	2/31 (6%)
Suchy	1992	58	autopsy material, not specified	dot-blot hybridization	15 (26%)	0 (0%)	0 (0%)	0 (0%)
Weber	2006	36	liver	seq	14 (39%)	0 (0%)	0 (0%)	0 (0%)
Zauber	2003	42	diverse, 93% lymph node, 5% liver	SCCP analysis + seq	22 (52%)	0 (0%)	0 (0%)	0 (0%)
Overall		892	All sites	All methods	345/849 (41%)	39/345 (11%)	35/504 (7%)	76/892 (9%)
		276	Liver	All methods	84/233 (36%)	8/84 (10%)	11/149 (7%)	21/276 (8%)
		129	Lymph nodes	All methods	65/129 (50%)	18/65 (28%)	14/64 (22%)	32/129 (25%)

Abbreviations: ASO = allele-specific oligonucleotides; ASPCR = allele-specific polymerase chain reaction; M = metastasis; pts = patients; PT = primary tumor; qPCR = quantitative PCR; RFLP = restriction fragment length polymorphism; SSCP = single strand conformational polymorphism; seq = sequencing

* Total number of cases not specified

concordant *KRAS* status between lymph node metastases and the primary tumor in three patients. The *KRAS* status of the lymph nodes in the other three patients showed heterogeneity, of which at least one lymph node metastasis showed a different *KRAS* status compared with the primary tumor. However, this explains the discordance between the primary tumor and liver metastasis only in one patient (Table 3).

DISCUSSION

This is the first adequately powered study in CRC that compares *KRAS* mutation status between primary tumors and their corresponding liver metastases. We showed that tissue from the primary tumor can reliably be used for *KRAS* mutation testing in order to select patients for anti-EGFR therapy.

We observed a concordant *KRAS* mutation status in 96.4% of 305 paired samples of colorectal tumors and liver metastases. However, the difference in *KRAS* status was not clinically relevant in 5 of the 11 patients with discordant results, because both primary tumor and metastasis had a different *KRAS* mutation. Given the high statistical power of our analysis, we were able to obtain a highly accurate estimate of the level of discordance that enabled us to conclude that the level of discordance was 2.0%. The high rate of concordance is in agreement with the notion that *KRAS* mutations are considered as early driving events in CRC progression, and associated with the growth of small adenoma to clinically significant size.²⁹ Therefore, *KRAS* mutation status is expected to be equal in both primary tumors and metastases.¹⁰

The previously reported lower concordance levels between primary tumors and metastases are most likely due to bias caused by false-negative results in underpowered studies. We calculated that 304 paired cases were needed to reliably exclude a rate of discordance of > 5%, while previous studies included only 10 to 110 patients (Table 4). Moreover, in these studies metastases of different sites were compared with the primary tumor. As the molecular patterns may differ between metastatic sites¹⁰, more reliable results are obtained when *KRAS* mutation status is tested more rigorously for each metastatic site. The liver is the predominant site of metastases in the majority of metastatic CRC patients, therefore the results of our large series of 305 liver metastases provide a solid reference for clinical decision making as to anti-EGFR therapy. Another issue is the fact that *KRAS* testing is technically not as straightforward as is often assumed. Several quality assurance systems are now in place, and the first 'round robin' test indicates that at least 30% of the experienced pathology laboratories fail to pass the threshold level of the quality assurance programs.³⁰ Other important facts about *KRAS* testing are the correct evaluation of the amount of tumor tissue in the sample and the

sensitivity of testing methods. In a previous study we demonstrated in > 500 samples that both sequencing and real-time PCR are reliable methods.³¹

A discordant *KRAS* status between the primary tumor and metastasis was observed in a small number of patients (3.6%). In these cases tumor cells may have departed the primary lesions prior to the acquisition of a fully malignant phenotype to undergo somatic mutations or deletions at a distant site.¹⁰ Another explanation for the discordant results may be heterogeneity of *KRAS* status within the primary tumor, although this was the case in only a small number of patients. Lastly, a discordance may in theory be explained by metastases from a non-detected second primary.

Previously published data showed that a considerable fraction (25%, Table 4) of colorectal lymph node metastases does not resemble the primary tumor in terms of *KRAS* mutation status. In 5 of the 25 lymph node metastases that we tested the *KRAS* status was not concordant with the primary tumor, which is consistent with the literature (Table 4). Therefore lymph node metastases do not seem suitable for determination of the *KRAS* mutation status of colorectal carcinomas. Discordance in *KRAS* mutation status might be due to clonal selection during the process of metastasis, however heterogeneity in lymph node metastases could explain this discordance in only one patient.



Eight different *KRAS* mutation types were observed in our study, of which Gly12Asp showed the highest frequency. Five patients (1.6%) harbored different *KRAS* mutation types in the primary tumor compared with the metastases. This confirms the findings of Cejas *et al.*²⁰ and Albanese *et al.*¹⁶, who reported a small number of patients (4 and 7% respectively) with different mutation types between primary tumors and metastases. A different *KRAS* mutation type between primary lung adenocarcinomas and corresponding lymph node metastases was also observed in only 1% of the patients.³² Currently, all patients with a *KRAS* mutation are excluded from treatment with anti-EGFR antibodies, independently of the mutation type. However, a recent paper indicated that codon 13 mutated tumors may be sensitive to cetuximab treatment.³³ As we observed a low frequency in *KRAS* mutation type discrepancies between primaries and metastases, this is not of clinical importance in selecting patients for anti-EGFR therapy.

In conclusion, we demonstrated a high level of concordance of 96.4% between primary tumors and liver metastases, which for clinical purposes to select CRC patients for anti-EGFR therapy was even higher with 98%. The implication of these results for general oncology practice is that both tissue of primary tumor or liver metastasis may be used for *KRAS* mutation testing. The results of our study are only valid for liver metastases and cannot be extrapolated to other metastatic locations. Furthermore, we demonstrated that discordance of test results between primary tumor and metastases cannot account for the failure rate of anti-EGFR therapy in patients with *KRAS* wild-type tumors. Therefore novel predictive markers in addition to *KRAS* and *BRAF* mutation status are warranted.

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Chapter 6 **Beyond *KRAS* mutation status: influence of *KRAS* copy number status and microRNAs on clinical outcome to cetuximab in metastatic colorectal cancer patients**

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ABSTRACT

BACKGROUND: *KRAS* mutation is a negative predictive factor for treatment with anti-epidermal growth factor receptor (EGFR) antibodies in metastatic colorectal cancer (mCRC). Novel predictive markers are required to further improve the selection of patients for this treatment. We assessed the influence of modification of *KRAS* by gene copy number aberration (CNA) and microRNAs (miRNAs) in correlation to clinical outcome in mCRC patients treated with cetuximab in combination with chemotherapy and bevacizumab.

METHODS: Formalin-fixed paraffin-embedded primary tumor tissue was used from 34 mCRC patients in a phase III trial, who were selected based upon their good ($n = 17$) or poor ($n = 17$) progression-free survival (PFS) upon treatment with cetuximab in combination with capecitabine, oxaliplatin, and bevacizumab. Gene copy number at the *KRAS* locus was assessed using high resolution genome-wide array CGH and the expression levels of 17 miRNAs targeting *KRAS* were determined by real-time PCR.

RESULTS: Copy number loss of the *KRAS* locus was observed in the tumor of 5 patients who were all good responders including patients with a *KRAS* mutation. Copy number gains in two wild-type *KRAS* tumors were associated with a poor PFS. In *KRAS* mutated tumors increased miR-200b and decreased miR-143 expression were associated with a good PFS. In wild-type *KRAS* patients, miRNA expression did not correlate with PFS in a multivariate model.

CONCLUSIONS: Our results indicate that the assessment of *KRAS* CNA and miRNAs targeting *KRAS* might further optimize the selection of mCRC eligible for anti-EGFR therapy.

INTRODUCTION

Recent advances in our understanding of the specific signalling pathways of cancer cells have introduced targeted therapy into treatment regimes for patients with metastatic colorectal cancer (mCRC). Antibodies against the epidermal growth factor receptor (EGFR), cetuximab and panitumumab, have shown a survival benefit in mCRC patients with *KRAS* wild-type tumors both as monotherapy^{1,2} and when added to chemotherapy.^{3,4} Patients with a tumor harboring a *KRAS* codon 12 or 13 mutation are resistant to anti-EGFR therapy.^{1,5} Therefore the use of these antibodies is restricted to patients with *KRAS* wild-type tumors. However, within this subset not all patients respond to this treatment, and therefore additional predictive markers are needed. We have previously excluded a discordance in *KRAS* mutation status between the primary tumor and corresponding metastases as an explanation for the heterogeneous response rate in patients with *KRAS* wild-type tumors.⁶ In routine practice, *KRAS* mutations in codons 12 and 13 are tested, which comprise approximately 96 % of the observed *KRAS* mutations.⁷ Recent data suggest that a codon 13 *KRAS* mutation has a distinct clinical behavior and is not associated with cetuximab resistance.⁸ Whether other *KRAS* mutations (like codon 61) result in similar resistance to EGFR monoclonal antibodies remains speculative.⁹ A mutation in the *BRAF* oncogene occurs in approximately 10% of mCRC patients and is restricted to *KRAS* wild-type tumors, and was first shown to have a negative predictive value for anti-EGFR therapy.¹⁰ Subsequently, we have shown that a *BRAF* mutation predominantly has a strong negative prognostic value.¹¹ Other biomarkers in the PI3K and RAS/MAPK pathways¹²⁻¹⁶, ligands to the EGFR^{17,18}, and germline single nucleotide polymorphisms¹⁹⁻²¹ have not yet shown a predictive value that can be used in clinical practice.

Point mutations in the *KRAS* oncogene lead to a significantly increased RAS-GTPase activity, ultimately resulting in the stimulation of cell proliferation and the inhibition of apoptosis via the RAS/MAPK pathway.²² However, in addition to oncogenic mutations, copy number changes of the *KRAS* gene or posttranslational factors may also be involved in the regulatory mechanism of RAS-GTPase activity. Copy number aberrations (CNA) occur throughout the tumor genome and are an important mechanism in colorectal cancer development.²³ Genome-wide studies in mCRC patients have identified loci that are associated with a poor prognosis²⁴ and with the prediction of response to chemotherapy.²⁵ However, little is known about the prevalence and effect of CNA of the *KRAS* locus on chromosome 12p12.1. By using a TaqMan-based *KRAS* copy number assay, *KRAS* amplifications were observed in approximately 2% of the 106 investigated colorectal primary tumors.²⁶ In CRC cell lines, gains of the *KRAS* locus were shown to be associated with an eleven-fold increase in RAS-GTPase activity, which is comparable with the twelve-fold increase caused by a codon 12 or 13 mutation.²⁷ However, no data have

been reported on CNA affecting the *KRAS* locus and their possible association with response to cetuximab.

In recent years, a rapidly expanding interest has manifested on microRNAs (miRNAs). These single stranded RNAs of 19–23 nucleotides regulate gene expression by translational inhibition or mRNA degradation via imperfect base pairing to the 3′-untranslated region (3′UTR) of their target mRNAs.²⁸ MiRNAs are involved in the development of human cancer, and in case of dysregulation they can act either as oncogenes or tumor suppressors, depending on their target genes.²⁹ Recently several miRNAs were identified that target *KRAS*, resulting in the suppression of cancer development.^{30–33} *KRAS* contains multiple let-7 complementary sites, allowing the let-7 family of miRNAs to act as a tumor suppressor by regulating the *KRAS* mRNA.^{30,31} Also mir-18a and miR-143 directly recognize *KRAS*, and downregulation of these miRNAs accelerates tumorigenesis by reversal of *KRAS* suppression.³² The targeting effect of miR-18a on *KRAS* has been demonstrated in colon cancer cell lines irrespective of *KRAS* mutation status.³³ MiRNAs interfering with the RAS-signaling pathway may have predictive value or may even serve as targets for treatment. Currently no data are available on the clinical relevance of miRNAs involved in *KRAS* activity in patients treated with cetuximab.

In this study we analyzed the *KRAS* copy number status and the expression of miRNAs targeting *KRAS* in relation to clinical outcome in mCRC patients treated with first-line cetuximab-containing therapy.

MATERIAL AND METHODS

Patients

The patients included in this study participated in the CAIRO2 trial (CKT0 2005–02; ClinTrials.gov NCT00208546) of the Dutch Colorectal Cancer Group (DCCG).³⁴ In this multicenter phase III trial, 755 mCRC patients were randomized between first-line treatment with capecitabine (1000mg/m² bid.), oxaliplatin (130 mg/m²), and bevacizumab (7.5 mg/kg), or the same schedule with the addition of weekly cetuximab (250 mg/m², after initial 400 mg/m²). Translational research on tumor tissue was part of the informed consent procedure. The primary end point of the study was progression free survival (PFS), and secondary end points were overall survival, response rate, and toxicity. The median PFS in patients treated with cetuximab was 9.4 months (95 % CI 8.4–10.5 months), which was significantly shorter than the PFS of patients treated in the group without cetuximab (median PFS 10.7 months, 95 % CI 9.7–12.3 months, *p*=0.01). Patients in the cetuximab-group with a *KRAS* mutated tumor had a significantly

decreased median PFS compared to patients with a *KRAS* wild-type tumor (8.1 versus 10.5 months, respectively, $p = 0.04$).

For the current analysis we selected patients who had been randomized to the cetuximab treatment arm, received at least three treatment cycles, did not discontinue treatment for other causes than disease progression, had a normal serum lactate dehydrogenase at randomization, and of whom formalin-fixed paraffin-embedded (FFPE) material of the primary tumor as well as normal tissue was available. Patients with a rectal carcinoma and patients who had received preoperative radiotherapy on the pelvis have been excluded for these analyses. From this group the 17 best and 17 worst responding patients were selected based on both extremes of the PFS time. Throughout the article the terms good and poor responders are used, which does not apply to response according to RECIST, but to the patients with the longest and shortest PFS on cetuximab-based treatment. This outcome parameter was chosen for the current study because it is the best reflection of the clinical trial upon which this analysis is based. Next, especially with respect to targeted agents PFS appears to be superior of response rate in terms of clinical outcome.

DNA extraction and mutation analysis

Genomic DNA was extracted from 4–8 manually micro dissected 50 μm sections of FFPE tissue as previously described.³⁵ DNA concentration was determined using the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies Inc., Wilmington, USA). DNA quality was assessed by performing a multiplex PCR using 4 primer sets, resulting in fragments of 100, 200, 300 and 400 base pairs.³⁶ The *KRAS* mutation status³⁵ and *BRAF* mutation status^{11,12} were assessed by sequencing analysis as previously described.

Assessment of the *KRAS* gene copy number and data analysis

High-resolution genome-wide DNA copy number profiles were generated by array-based comparative genomic hybridization (array CGH) using 720 k Whole-Genome Tiling CGH arrays (Roche NimbleGen Inc., Madison, USA). Optimal signal-to-noise ratios were obtained by hybridizing test (tumor) and reference (normal colon) DNA of similar quality, which was determined by giving similar yield in a Bioscore Screening and Amplification kit (ENZO diagnostics Inc., Farmingdale, USA). For hybridization, 500 ng of amplified DNA from test and reference samples were labelled with Cy3 and Cy5, respectively, using random-primed labelling (Bioprime genomic DNA labelling kit, Invitrogen, Breda, the Netherlands), and hybridized for 48 hours at 42°C using a MAUI hybridization system (Biomicro Systems, Salt Lake City, USA). After washing, arrays were scanned in an Axon Genepix 4200AL microarray scanner. The NimbleScan 2.4 software package (NimbleGen Systems Inc., Madison, USA)

was used to calculate \log_2 ratios after performing spatial correction, normalization and a 25 kb average smoothing window on the data. Further data interpretation and CNA calling was done with Nexus Copy Number 5.0 software (Biodiscovery, El Segundo, USA) using the Rank Segmentation Algorithm. In 26 patients, hybridizations were performed against normal DNA from the same patient to normalize for germline copy number changes. In the other 8 cases, germline copy number changes were excluded using both public (<http://projects.tcag.ca/variation/>) and private CNA databases. The cut-off value for gene copy number gain and loss were manually set for each sample to adjust for differences in signal strength and incorrectly centered baselines.

Prevalence of *KRAS* locus gene copy number changes

In order to assess the clinical relevance in terms of prevalence of *KRAS* gene copy number changes in mCRC patients, we assessed the *KRAS* gene copy number status in FFPE primary tumor tissue of 225 unselected mCRC patients who participated to our previous phase III study which did not involve the use of targeted agents.³⁷ In these patients a 250 k oligonucleotide array CGH was performed as previously described.²⁵

Multiplex Ligation-dependent Probe Amplification (MLPA)

MLPA was performed according to the manufacturer's instruction using the SALSA P145-A2 kit (MRC Holland, Amsterdam, the Netherlands), containing 40 probes including the 12p12.1 *KRAS* probe. Briefly, 200 ng DNA was denatured and allowed to hybridize for 16 h at 60°C in a thermocycler. Then SALSA Ligase-65 enzyme was added and ligation was allowed at 54°C. After heat inactivation of the ligase enzyme at 98°C, primers, dNTPs and polymerase were added and PCR amplification was performed for 35 cycles (60s at 95°C, 30s at 60°C, and 90s at 72°C). Reactions were performed on a PTC 200 thermal cycler (MJ Research Inc., Waltham, Massachusetts, USA). One microliter of PCR product was analyzed by capillary electrophoresis on an ABI 3730 Analyzer (Applied Biosystems), and quantitative data were obtained by Genemapper analysis (Applied Biosystems).

MLPA data analysis

For each tumor sample, the peak area of the 12p12.1 and reference probes were determined in duplicate for further analysis. The reference peak area was obtained from blood samples from three different individuals, each of which were analyzed at least two times independently. In every sample, for every probe, a tumor to normal DNA copy number ratio was calculated by dividing the median area under the peak for the 12p12.1 probe by the value for the reference

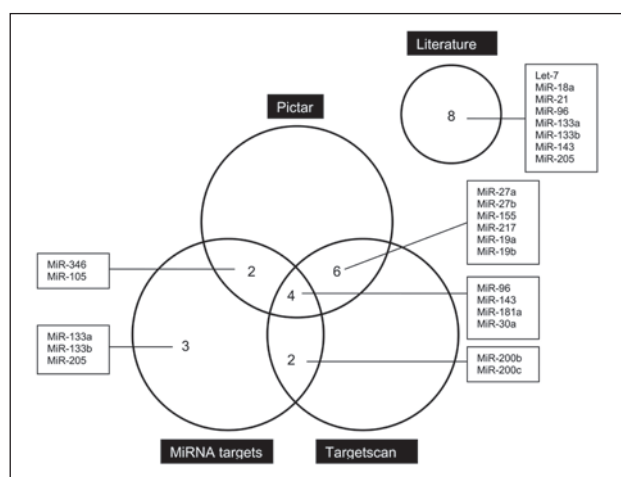
DNA. Subsequently, all ratios were normalized by setting the median tumor to normal DNA copy number ratio of the reference genes in the probe mixture to 1.0. A ratio lower than 0.8 was considered a loss and a ratio higher than 1.2 a gain.

MiRNA selection

Selection of miRNAs regulating *KRAS* was performed using PicTar (<http://pictar.mdc-berlin.de/>), TargetScan (<http://www.targetscan.org/>) and miRNA targets (<http://cbio.mskcc.org/mirna-viewer/>). Venn diagram analysis was used to select 14 miRNAs who were identified by at least two algorithms (Additional file1). In addition to the prediction programs we also selected six extra miRNAs (Let-7, miR-18a, miR-21, miR-133a, miR-133b, miR-205) which have been shown to target *KRAS* in previous studies^{30,33,38}, resulting in a total test series of 20 miRNAs. Two Taqman microRNA assay were not available (mir-18a, mir-200c), resulting in 18 miRNAs to analyze.

Total RNA extraction, miRNA reverse transcription and real-time PCR

Total RNA was isolated from FFPE tissue of 34 primary tumors and matched normal tissue using the RecoverAll™ Total Nucleic Acid Isolation Kit (Applied Biosystems, Foster city, USA). In brief, four tissue slices of 20 µm were micro dissected and incubated with 100% xylene at 50°C to remove paraffin excess, followed by ethanol washes. Proteins were degraded by protease at 50°C and 80°C. The RNA was extracted followed by nuclease digestion. Total RNA



Additional figure 1. Selection of MiRNAs that were identified by multiple algorithms.

quantity and quality were determined using the Nanodrop 26 ND-1000 spectrophotometer (Nanodrop Technologies Inc., Wilmington, USA).

To determine the expression levels of miRNAs, Taqman miRNA assays directed to seventeen miRNAs and the endogenous reference gene (RNU 6B) were used following the manufacture's protocol (Applied Biosystems, Foster City, USA). Firstly, cDNA was synthesized in duplicate from total RNA using miRNA specific stem loop primers. Reverse transcriptase reactions were conducted using 10 ng total RNA, 1 mM dNTPs, 50 U MultiScribe™ Reverse Transcriptase, 1 x RT buffer, 3.8 U RNase inhibitor and 1 x Taqman® MicroRNA RT Primer (Applied Biosystems, Foster city, USA). The 15 µl reactions were incubated at 16°C for 30 minutes at 42°C for 30 minutes and at 85°C for 5 minutes.

Secondly, the quantitative PCR was performed in which the total mixture of 20 µl included 1.33 µl RT product (1:5 diluted from RT reaction), 1 x Taqman® Universal PCR Master Mix (No AmpErase® UNG, Applied Biosystems, Foster City, USA) and 1 x the dedicated primer and probe mix. The reactions were incubated in a 96-well optical plate at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and at 60°C for 1 minute. All reactions were carried out in duplicate in a 7500 Real Time PCR System (Applied Biosystems, Foster City, USA). The threshold cycle (Ct) was defined as the fractional cycle number at which the fluorescence passes the fixed threshold. Relative quantification of miRNA expression was calculated using the $\Delta\Delta C_t$ method as described previously.³⁹

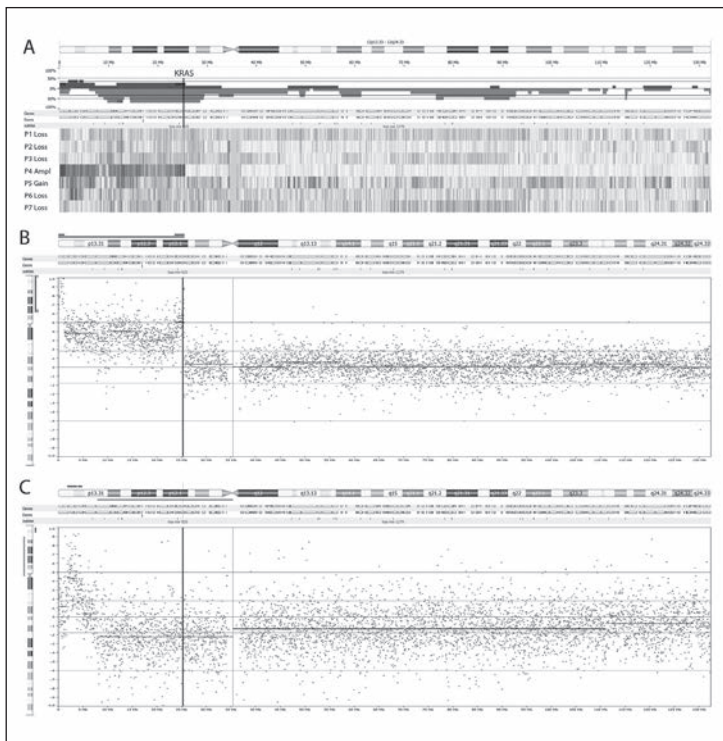
Statistical analysis

PFS was defined as the interval from the date of randomization to the date of first documented disease progression or death, whichever occurred first. Statistical differences of clinical and pathological parameters between good and poor responders were evaluated using the Student's t-test, Pearson's χ^2 test or Fisher's exact test where appropriate. The miRNA expression in colorectal tumors was described by the relative quantity (RQ) of the target miRNA, normalized in respect to RNU6B and relative to matched normal tissue. Box plots were used to appreciate the descriptive statistics of the data. Differences in expression of the target miRNA between good and poor responders were evaluated on the log scale ($\Delta\Delta C_t$ scale) to obtain normally distributed data. The Student's t-tests was used in exploratory analyses on the miRNA expression in relation to response and to *KRAS* mutation status. When focusing on the actually observed PFS, we investigated by Cox regression analysis the influence of each miRNA on PFS, using *KRAS* mutation status, the interaction term between miRNA and *KRAS* mutation, and differentiation grade as covariates. Due to the limited number of patients and the ensuing risk for overfitting, it was not possible to assess the influence of all miRNAs together (i.e. correct the influence of miRNA for each other), nor to correct for other baseline characteristics.

RESULTS

Patients

Of the 34 patients selected for this analysis, the median PFS was 22.5 months (range 14.8-39.8 months) in the 17 good responders, and 6.0 months (range 2.3-7.2 months) in the 17 poor responders. Clinical and pathological characteristics of the primary tumor were well balanced between the 17 good and 17 poor responders. Only poor differentiation grade of the primary tumor was more frequently observed in the poor responders.



Additional figure 2. Heat map representation and individual array CGH plots of patients with *KRAS* copy number aberrations **A:** Heat map representation of the 7 patients with CNA of the *KRAS* locus. Each row represents a patient with a CNA of the *KRAS* locus (loss, gain, amplification). Whole chromosome 12, containing the *KRAS* locus, is depicted on the horizontal axis. **B:** Amplification of a genomic region in 12p12.1 detected by array CGH in two patients, as confirmed by MLPA. The DNA log₂ ratios and whole chromosome 12 are represented on the vertical and horizontal axis, respectively. **C:** Array CGH plot of chromosome 12 of a patient with a deletion of *KRAS*, which could not be validated using MLPA, but was detected by the Nexus copy number algorithm. Most of the genomic deletions detected by array CGH appeared to be present subclonal, below the detection threshold of MLPA. The DNA log₂ ratios and whole chromosome 12 are represented on the vertical and horizontal axis, respectively. Abbreviation: ampl = amplification.

A *KRAS* mutation was demonstrated in the primary tumor of 15 patients (6 good responders and 9 poor responders), and *KRAS* wild-type in the primary tumor of 19 patients (11 good responders and 8 poor responders). *KRAS* codon 12 mutation was observed in 14 patients, and

Table 1. Clinical and histopathological characteristics of patients and their respective tumors

		All eligible patients n = 34	Good responders n =17	Poor Responders n =17	P-value
Age	Mean	58.6	58.0	59.2	0.07
Gender	Female	14 (41%)	5 (29%)	9 (53%)	0.30
	Male	20 (59%)	12 (71%)	8 (47%)	
Number of metastatic sites	1	17 (50%)	9 (53%)	8 (47%)	0.60
	>1	17 (50%)	8 (47%)	9 (53%)	
WHO PS	0	23 (68%)	11 (65%)	12 (71%)	0.71
	1	11 (32%)	6 (35%)	5 (29%)	
Site of primary tumor	Colon	22 (65%)	11 (65%)	11 (65%)	0.90
	Rectosigmoid	12 (35%)	6 (35%)	6 (35%)	
T stage	1-2	4 (12%)	2 (12%)	2 (12%)	0.49
	3	21 (62%)	12 (71%)	9 (53%)	
	4	9 (26%)	3 (18%)	6 (35%)	
N stage	0	9 (26%)	4 (24%)	5 (29%)	0.61
	1	8 (24%)	4 (24%)	4 (24%)	
	2	14 (41%)	9 (53%)	5 (29%)	
	Unknown	3 (9%)	0	3 (18%)	
Differentiation grade	Good	1 (3%)	1 (6%)	0	0.02
	Moderate	23 (68%)	15 (88%)	8 (47%)	
	Poor	10 (29%)	1 (6%)	9 (53%)	
<i>BRAF</i> mutation status	Wild-type	30 (88%)	16 (94%)	14 (82%)	0.29
	Mutant	4 (12%)	1 (6%)	3 (18%)	
<i>KRAS</i> mutation status	Wild-type	19 (56%)	11 (65%)	8 (47%)	0.30
	Mutant	15 (44%)	6 (35%)	9 (53%)	
<i>KRAS</i> mutation type	Codon 12	14 (93%)	6 (100%)	8 (89%)	0.40
	Codon 13	1 (7%)	0	1 (11%)	
PFS (months)	Median (range)	11.0 (2.3-39.8)	22.5 (14.8-39.8)	6.0 (2.3-7.2)	<0.0001

Abbreviations: WHO = World Health Organization; PS = Performance status;

PFS = progression-free survival

one poor responder had a codon 13 mutation. Of the *KRAS* wild-type patients, 4 had a *BRAF* mutated tumor (1 good responder and 3 poor responders) (Table 1).

12p12.1 copy number changes in good and poor responders

By using high resolution array CGH, two copy number gains (of which one amplification) and 5 losses were detected at the 12p12.1 locus where *KRAS* is localized (Additional file2). Both copy number gains, which were confirmed by MLPA, were observed in poor responders with a *KRAS* wild-type tumor. Of these tumors one sample contained a gain of the complete p-arm of chromosome 12 and the other sample contained a high copy number gain of a region including the *KRAS* locus.

A 12p12.1 copy number loss, detected by array CGH, was observed in the tumor of 5 patients with a good response. One tumor contained a loss of the whole chromosome, three tumors included a loss of the short arm of the chromosome and one tumor contained a loss of a 27.5 Mb region of the short arm of chromosome 12 including the *KRAS* locus (Additional file2). Of these 5 tumors with loss of the 12p12.1 locus, 2 tumors harbored a *KRAS* mutation, and one tumor had a *BRAF* mutation, suggesting that the mechanism of gene copy number loss is independent of the *KRAS* and *BRAF* mutation status (Figure 1).

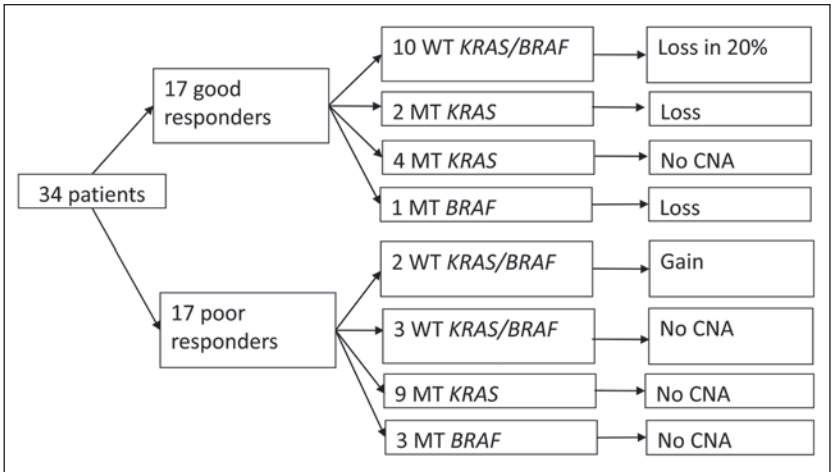


Figure 1. 12p12.1 copy number changes in good and poor responders according to the *KRAS* mutation status. Abbreviation: CNA = copy number aberration.

12p12.1 gene copy number changes in a control group of mCRC patients

In an unselected group of 222 mCRC patients from our previous trial with comparable baseline characteristics³⁷, the prevalence of 12p12.1 copy number changes was assessed. In this group three amplifications (1.4%), 32 copy number gains (14.4%), and 12 losses (5.4%) of the 12p12.1 locus were observed. There was no effect of *KRAS* copy number gain or loss on prognosis in these patients treated with first-line chemotherapy without cetuximab ($p=0.97$ and $p=0.75$, respectively, data not shown).

MiRNA expression in good and poor responders

To assess the role of miRNA expression in relation to clinical outcome, the expression levels of 18 miRNAs targeting *KRAS* were determined by real-time RT-PCR in 32 primary colorectal tumors relative to their matched normal tissue. Two patients (1 good and 1 poor responder) were not accessible for miRNA expression due to an insufficient RNA amount in normal

Table 2. MiRNA expression in good versus poor responders

	Good responders			Poor responders			RQ good versus poor	P-value
	Mean ddCt	SE	RQ	Mean ddCt	SE	RQ		
MiR-27b	-0.98	0.77	1.98	-0.92	0.64	1.90	1.04	0.80
MiR-105	-1.04	2.80	2.05	0.57	3.15	0.68	3.01	0.21
MiR-155	-0.66	1.25	1.58	-0.19	0.89	1.14	1.39	0.24
MiR-346	0.32	1.52	0.80	0.57	1.14	0.67	1.19	0.60
MiR-181a	-1.64	0.85	3.11	-1.27	0.75	2.42	1.29	0.21
MiR-19a	-3.39	1.84	10.45	-2.82	1.55	7.04	1.48	0.35
MiR-200b	-0.57	0.92	1.49	0.05	1.42	0.97	1.54	0.15
MiR-27a	-2.10	0.98	4.27	-2.00	0.82	4.00	1.07	0.77
MiR-30a	-0.81	0.86	1.75	-0.66	0.88	1.58	1.11	0.65
Let-7a	-0.67	0.79	1.59	-0.39	0.70	1.31	1.21	0.30
MiR-21	-3.16	1.16	8.92	-2.75	0.79	6.75	1.32	0.26
MiR-96	-4.56	1.32	23.59	-3.81	1.40	14.04	1.68	0.13
MiR-143	-0.73	1.35	1.66	-1.76	1.55	3.38	0.49	0.07
MiR-217	-2.91	3.09	7.53	-1.64	3.10	3.12	2.41	0.30
MiR-133a	0.91	1.69	0.53	0.33	1.79	0.79	0.67	0.36
MiR-133b	0.98	1.74	0.51	0.39	2.23	0.76	0.67	0.41
MiR-19b	-2.95	1.71	7.73	-2.45	1.37	5.45	1.42	0.37

Abbreviations: SE = standard error ; RQ = relative quotient

mucosa. MiR-205 expression was undetectable in both tumor and normal mucosa, therefore 17 miRNAs were included in our final analysis. By using NormFinder⁴⁰ and GeNorm⁴¹, the use of RNU6B as a reference gene was justified.

The expression level of 14 miRNAs showed a trend towards a higher expression in patients with a good response compared to patients with a poor response, however this trend was not statistically significant. MiR-143, miR-133a and miR-133b expression was decreased in patients with a good response, of which miR-143 showed a relative expression in good versus poor responders of 0.49 ($p = 0.07$) (Table 2).

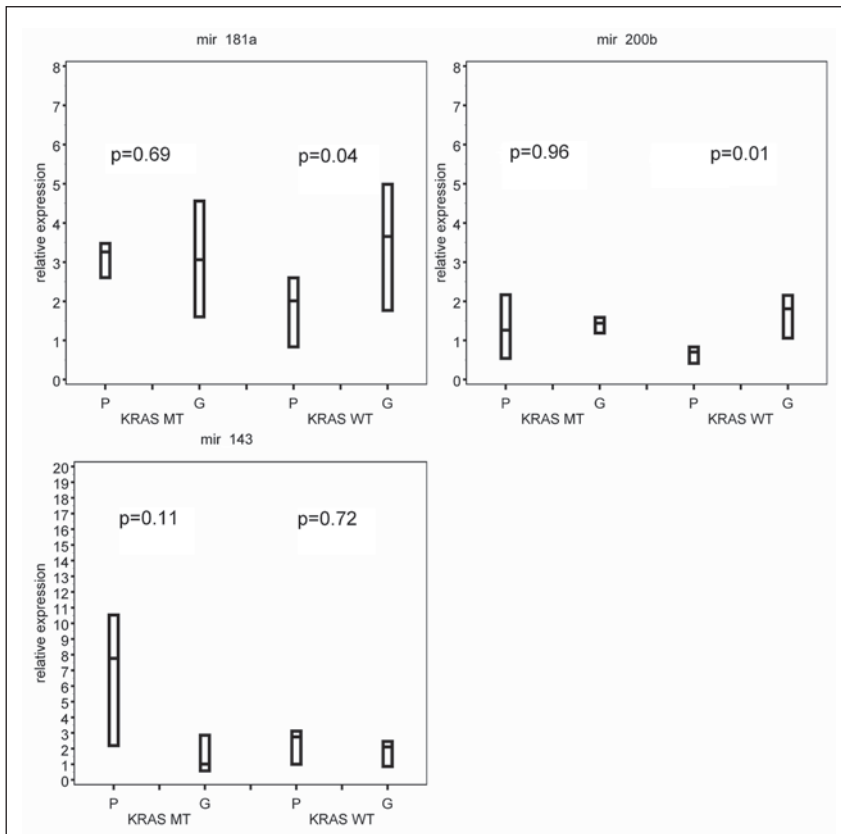


Figure 2. Box plots of the expression levels of miR-181a, miR-200b and miR-143 in mCRC patients according to clinical outcome and KRAS mutation status. Abbreviations: G = good responders; P = poor responders; MT = mutant; WT = wild-type

MiRNA expression in good and poor responders according to *KRAS* mutation status

In patients with a wild-type *KRAS* tumor, the expression level of miR-181a showed a 1.87-fold increase in good responders compared to poor responders ($p = 0.04$), which was not observed in patients with mutated *KRAS* tumors (0.91-fold increase, $p = 0.69$). A higher expression of miRNAs in wild-type *KRAS* good responders compared with wild-type *KRAS* poor responders was also observed for miR-200b (2.48-fold increase, $p = 0.01$) and miR-21 (1.66-fold increase, $p = 0.06$).

A difference between the expression of miR-143 in good versus poor responders was more obvious in mutated *KRAS* tumors. The relative expression level of miR-143 showed a 0.30 fold increase in mutated *KRAS* good responders versus mutated *KRAS* poor responders ($p = 0.11$) (Figure 2).

Multivariate model of PFS in relation to miRNA expression and *KRAS* mutation status

Each miRNA was analyzed individually together with differentiation grade as a covariate for PFS in wild-type *KRAS* and mutated *KRAS* patients treated with first-line cetuximab-containing therapy (Table 3). Differentiation grade was used as a covariate in the Cox regression model because this pathological feature is a well known prognostic factor and differentially distributed between good and poor responders.

Elevated expression of miR-200b was associated with a better PFS in patients with a mutated *KRAS* tumor (HR 0.56 (0.28-1.15); $p = 0.10$). This trend was not present in patients with a wild-type *KRAS* tumor. Surprisingly, increased expression of miR-143 resulted in a shorter PFS in patients with a mutated *KRAS* tumor (HR 1.59 (1.01-2.50); $p = 0.04$). The hazard ratio for PFS was not influenced by miR-143 expression in wild-type *KRAS* tumors.

DISCUSSION

We demonstrated that regulation of the *KRAS* oncogene at several levels might affect clinical outcome in a selected group of cetuximab-treated mCRC patients treated in a phase III trial.³⁴ Copy number loss of the *KRAS* locus was restricted to good responders, whereas a copy number gain was associated with a poor PFS in patients with wild-type *KRAS* tumors. Increased expression of miR-200b that targets *KRAS* was associated with improved PFS in patients with

a mutated *KRAS* tumor. Surprisingly, decreased miR-143 expression was correlated with improved PFS in these patients.

The predictive strength of *KRAS* mutation status stresses the importance of RAS-GTPase activity for the response to cetuximab. Therefore, other regulatory mechanisms of RAS-GTPase activity are obvious novel candidate markers. CNA of the *KRAS* locus occur independently of the *KRAS* mutation status in a considerable percentage of colorectal tumors (21.2%) as assessed in a large and unselected mCRC population. Previously, it has been shown that *KRAS* copy number gains are correlated with increased RAS-GTPase activity in colorectal cell lines and with worse clinical outcome in lung adenocarcinomas.²⁷ Our results suggest that *KRAS* copy number gains are associated with worse clinical outcome in wild-type *KRAS* mCRC patients who are treated with a cetuximab-containing first-line regimen. This influence of *KRAS* copy number gain on prognosis was absent in mCRC patients treated without cetuximab, suggesting a predictive effect on cetuximab response. The correlation between miRNAs targeting *KRAS* and PFS was absent in wild-type *KRAS* patients. Inhibition of *KRAS* translation by miRNAs is probably only relevant when the *KRAS* expression levels are high, which is not the case in absence of an activating *KRAS* mutation.

KRAS mutations occur in approximately 38% of mCRC patients⁷, and these patients are currently excluded from treatment with anti-EGFR antibodies. However, in our selected good responders, 6 patients (35 %) had a *KRAS* mutated tumor. A recent publication showed that patients with codon 13-mutated tumors might benefit from cetuximab treatment.⁸ In the current series none of the good responders had a tumor with a *KRAS* codon 13 mutation. Our data show that the presence of *KRAS* copy number loss in two of the mutated *KRAS* mCRC patients might justify treatment with cetuximab. Decreased expression of *KRAS* caused by loss of gene copies in correlation with response to cetuximab has not been described earlier. Despite the limitations in sample size and concomitant treatment our results indicate that patients with *KRAS* copy number loss might benefit from treatment with an anti-EGFR antibody although their tumor is *KRAS* mutated.

Next, we demonstrated that increased expression of miR-200b was associated with improved PFS in mutated *KRAS* patients. We hypothesize that reducing *KRAS* protein levels in the presence of a mutation might improve clinical outcome in patients treated with cetuximab. *KRAS* is not the only target of the miR-200 family, miR-200b is also capable of reducing *ERRFI-1* mRNA and subsequent activation of EGFR.⁴² Adam et al. showed that increased expression of miR-200b facilitates optimal EGFR functionality, resulting in an efficient response of bladder cancer cells to cetuximab. To our knowledge, our results are the first data *in vivo* suggesting that in the presence of a *KRAS* mutation, an increased miR-200b expression is associated with an improved PFS in cetuximab-treated mCRC patients. Surprisingly a decreased expression of miR-143 was associated with improved PFS in patients with mutated *KRAS* tumors. MiR-143 is thought to inhibit *KRAS* translation and thereby to suppress tumor cell growth during tumor-

Table 3. A multivariate model in which each miRNA was analyzed individually together with differentiation grade as a predictor for PFS in wild-type *KRAS* and mutated *KRAS* patients treated with chemotherapy, bevacizumab and cetuximab

		Overall n = 32	<i>KRAS</i> wild-type n = 18	<i>KRAS</i> mutation n = 14	P-value
MiR-27b	HR (95% CI)	0.74 (0.38-1.42)	0.94 (0.36-2.42)	0.51 (0.20-1.32)	0.38
	p-value	0.36	0.89	0.16	
MiR-105	HR (95% CI)	1.04 (0.84-1.29)	0.95 (0.76-1.21)	1.22 (0.83-1.78)	0.26
	p-value	0.72	0.69	0.32	
MiR-155	HR (95% CI)	0.92 (0.62-1.35)	0.77 (0.38-1.53)	0.96 (0.60-1.51)	0.60
	p-value	0.66	0.45	0.85	
MiR-346	HR (95% CI)	0.83 (0.63-1.10)	0.85 (0.61-1.19)	0.72 (0.40-1.32)	0.63
	p-value	0.20	0.34	0.29	
MiR-181a	HR (95% CI)	0.75 (0.46-1.22)	0.70 (0.37-1.33)	0.94 (0.44-2.02)	0.57
	p-value	0.24	0.27	0.87	
MiR-19a	HR (95% CI)	0.96 (0.78-1.19)	1.03 (0.80-1.33)	0.74 (0.45-1.20)	0.23
	p-value	0.73	0.82	0.22	
MiR-200b	HR (95% CI)	1.03 (0.72-1.47)	0.90 (0.51-1.57)	1.78 (0.87-3.62)	0.18
	p-value	0.86	0.70	0.10	
MiR-27a	HR (95% CI)	0.74 (0.43-1.27)	0.69 (0.31-1.54)	0.71 (0.35-1.43)	0.96
	p-value	0.27	0.37	0.34	
MiR-30a	HR (95% CI)	0.70 (0.44-1.10)	0.77 (0.40-1.55)	0.70 (0.37-1.34)	0.86
	p-value	0.12	0.46	0.28	
Let-7a	HR (95% CI)	0.94 (0.59-1.50)	0.89 (0.50-1.59)	1.28 (0.46-3.58)	0.55
	p-value	0.80	0.68	0.63	
MiR-21	HR (95% CI)	0.89 (0.57-1.39)	1.15 (0.52-2.55)	0.81 (0.49-1.33)	0.47
	p-value	0.61	0.74	0.40	
MiR-96	HR (95% CI)	0.84 (0.59-1.20)	0.74 (0.45-1.22)	0.93 (0.55-1.58)	0.54
	p-value	0.33	0.24	0.79	
MiR-143	HR (95% CI)	0.73 (0.51-1.05)	0.92 (0.56-1.51)	0.63 (0.40-0.99)	0.26
	p-value	0.09	0.74	0.04	
MiR-217	HR (95% CI)	1.01 (0.87-1.16)	0.85 (0.61-1.19)	0.78 (0.56-1.08)	0.72
	p-value	0.95	0.34	0.13	
MiR-133a	HR (95% CI)	0.81 (0.63-1.05)	0.98 (0.68-1.43)	0.66 (0.41-1.06)	0.21
	p-value	0.12	0.92	0.09	
MiR-133b	HR (95% CI)	0.89 (0.70-1.13)	1.04 (0.73-1.49)	0.80 (0.55-1.17)	0.32
	p-value	0.32	0.83	0.25	
MiR-19b	HR (95% CI)	0.95 (0.75-1.20)	1.02 (0.77-1.34)	0.66 (0.36-1.23)	0.21
	p-value	0.65	0.91	0.19	

Abbreviation: HR = hazard ratio

igenesis.³² In an established tumor, modulation of *KRAS* by miR-143 may be differentially regulated which could possibly explain our findings. However, since miRNAs are capable of repressing over a hundred different mRNAs²⁸, miR-143 could also target mRNAs which may be relevant in response to capecitabine, oxaliplatin and bevacizumab. Previous studies on biomarkers have shown divergent results which stresses the importance of the results of our current hypothesis-generating study being confirmed in a larger, independent series of mCRC patients preferably treated with cetuximab monotherapy.

The patients used in this study were derived from a clinical trial, and the observed outcome is also influenced by the effect of the other agents used. The relative contribution of cetuximab to this outcome is therefore unclear. The phase III CAIRO2 trial showed that cetuximab plus chemotherapy and bevacizumab resulted in a significantly decreased median PFS compared to treatment with chemotherapy and bevacizumab alone. The explanation of this detrimental outcome is unclear⁴³, and complicates the interpretation of the current analysis. Excessive toxicity in the cetuximab group does not appear to be cause of these results. Negative interaction between the antibodies or between antibodies and chemotherapy might have influenced the outcome although preclinical observations supporting this hypothesis are not yet available. The interpretation of the current analysis is complicated by the detrimental outcome of the trial. Whether this outcome also affects the PFS in the good responders remains unclear.

In conclusion, the analysis of *KRAS* CNA and miRNAs targeting *KRAS* may optimize the selection of mCRC patients eligible for anti-EGFR therapy. Elevated expression of miR-200b, decreased miR-143 level and copy number losses may identify patients with mutated *KRAS* tumors who benefit from anti-EGFR therapy, whereas copy number gains in wild-type *KRAS* patients could predict resistance to cetuximab. Our results are relevant for the development of predictive biomarkers for anti-EGFR therapy, and suggest that the clinical effects of *KRAS* are the result of a complex interaction of several regulatory mechanisms beyond the *KRAS* point mutation status.

CONCLUSIONS

KRAS activity, an important regulator of response to anti-EGFR therapy, can be influenced by genetic and epigenetic regulation. CNA and specific miRNAs may provide important additional information to *KRAS* mutation status and their use could further improve the selection of mCRC patients for anti-EGFR therapy. The hypothesis-generating nature of our study urges for our results to be confirmed in larger series.

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Chapter 7 **MicroRNAs in colorectal cancer metastasis**

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ABSTRACT

Metastatic disease is the major cause of death in colorectal cancer (CRC) patients. The metastatic process is highly inefficient and comprises multiple sequential steps. While many genetic factors relevant in this process already have been identified, the epigenetic factors underlying each step still remain obscure. MicroRNAs (miRNAs) are key regulators in tumorigenesis, but their role in the development of cancer metastasis is poorly investigated. The majority of miRNAs involved in the metastatic process have been identified in breast cancer cell lines, and in CRC less data are available. We review the role of miRNAs in the metastatic pathway of CRC, including escape of apoptosis, epithelial-mesenchymal transition, angiogenesis, and invasion. Better understanding of the complex role of miRNAs in the development of CRC metastases may provide new insights which could be of therapeutic consequence.

INTRODUCTION

Colorectal cancer (CRC) is the second most common cause of cancer death worldwide. Approximately 50% of the patients diagnosed with CRC will die because of the complications of distant metastases. The formation of metastases is a multistep process, in which malignant cells disseminate from the primary tumor to colonize distant organs. This is a highly inefficient and complex process, which involves early steps of tumor cell invasion of the microenvironment, entering the bloodstream, survival during migration, and extravasation into distant organs. Subsequent steps including proliferation, induction of angiogenesis and evading apoptotic death, are crucial for colonization of the secondary site.¹ Tumor cells need to be proficient in all these processes in order to produce metastatic outgrowth. Therefore, each step in metastasis requires specific genetic and epigenetic changes.² Although research has identified multiple genes responsible for carcinogenesis, the (epi)genetic alterations that provide cancer cells the ability to metastasize are largely unknown.

MicroRNAs (miRNAs) are a family of small, highly conserved non-coding RNAs that post-transcriptionally regulate gene expression. Typically, pri-miRNAs are transcribed by RNA polymerase II in the nucleus. These pri-miRNAs are processed by Drosha and its cofactor DGCR8, yielding 60-70 nt precursor miRNAs (pre-miRNAs). Pre-miRNAs are transported into the cytoplasm, where they undergo processing by the RNase III enzyme Dicer, resulting in mature miRNAs which are incorporated into the RNA-induced silencing complex (RISC).³ MiRNAs act as endogenous suppressors of gene expression through imperfect binding of RISC to the 3'-untranslated region (3'-UTR) of target mRNAs, inducing either translational repression or mRNA degradation, yet mechanistic details of the function of each miRNA in translation inhibition and/or mRNA destabilization remain controversial. To date, more than 1000 human miRNAs have been identified (miRBase Sequence Database – Release 16) and each individual miRNA may control hundreds of target genes. More than 5300 human genes were predicted as miRNA targets, representing 30% of the human gene set.⁴ MiRNAs have important regulatory functions in basic biological processes that form the hallmarks of cancer, such as cellular differentiation, proliferation, migration, and apoptosis. Approximately 50% of the human miRNAs are located at chromosomal breakpoints and are therefore susceptible to dysregulation in human cancer.⁵ MiRNAs are involved in the pathogenesis of CRC, partly by regulating the expression of oncogenes and tumor suppressors and partly by functioning as oncogenes or tumor suppressors themselves.^{6,7} For example, the miR-135 family affects the Wnt signalling pathway by downregulating the tumor suppressor gene Adenomatous Polyposis Coli (*APC*), regardless of mutation status or promoter hypermethylation.⁸ MiR-137 and miR-342 both act as tumor suppressors and are frequently silenced by promoter hypermethylation in early stages

of CRC.^{9,10} Another potential tumor suppressive miRNA in CRC development is miR-143, who might regulate DNA methylation by targeting DNA methyltransferase 3A (*DNMT3A*).¹¹

An association between the dysregulation of miRNA expression and specific steps in the metastatic pathway is highly likely. The role of miRNAs in the development of metastases has been demonstrated in several different cancers, including hepatocellular carcinomas, head and neck cancer, and brain tumors.¹² However, the majority of miRNAs involved in metastasis have been identified in breast cancer. Because the expression of miRNAs are highly tumor and tissue-specific, there is a need for more information on the association of miRNAs and metastases in other common types of cancer, such as colorectal carcinomas. Most studies in CRC analyzed the association between miRNAs and the metastatic pathway using *in vitro* and mouse models. There are also tissue-based experiments in CRC patients, but the amount of data is still limited. In other human cancers, progress has already been made in therapeutic approaches based on miRNAs. Therefore, it is necessary to summarize the role of miRNAs in the development of metastasis, specifically in CRC.

We present a review on the relationship between miRNAs and the CRC metastatic pathway, including escape of apoptosis, epithelial-mesenchymal transition (EMT), angiogenesis, invasion, migration, and proliferation.

MIRNAS AND THE EVASION OF APOPTOSIS

Cells that have a permanent inactivation of tumor suppressive factors that induce cell-cycle arrest, senescence and apoptosis have a selective advantage and are more likely to metastasize. The multifunctional tumor suppressor gene *TP53* responds to DNA damage through the induction of cell cycle checkpoints, cellular senescence, and apoptosis.¹³ MiR-34a, a member of the miR-34 family, was identified as a direct downstream transcriptional target of *TP53* which is up-regulated after *TP53* induction by DNA-damaging agents.¹⁴ MiR-34a-responsive genes are highly enriched for those that regulate cell-cycle progression, cellular proliferation (*E2F*), apoptosis (*BCL2*), DNA repair and angiogenesis.¹⁴ Transfection of CRC cells with miR-34a induces senescence and apoptotic cell death.^{14,15} In human CRC, a significant subset (36%) of primary tumors show decreased miR-34a expression¹⁵, partially due to the presence of *TP53* mutations.¹⁶ Another mechanism of miR-34a downregulation is loss of chromosome 1p36 (the location of miR-34a), which is observed in 50% of the primary CRCs, 33% of the local recurrences, and 64% of the metastases.¹⁷ Primary CRC cells with metastatic ability may already contain a 1p36 deletion, or acquire such changes during the development of a metastatic lesion. Alternatively, epigenetic inactivation of the miR-34 family (consisting of

miR-34a, miR-34b, and miR-34c) by promoter hypermethylation is observed in the majority of cancer cell lines^{18, 19} and primary CRCs.¹⁹⁻²¹ The redundancy of methods by which tumor cells can cause down-regulation of miR-34a suggests an important role of this miRNA in CRC metastasis.

MiR-192, miR-194-2, and miR-215 are additional downstream targets of *TP53*, and all capable of inducing p21 expression and cell cycle arrest in a p53-dependent manner. They are capable of inducing partial cell cycle arrest, which indicates cell death. In addition, miR-192 and miR-215 are capable of inducing senescence to some extent but not as efficiently as miR-34a.²² Down-regulation of these miRNAs in CRC can partly be explained by loss or inactivation of *TP53*.²³ Additionally, for miR-194-2 a single nucleotide polymorphism (SNP) located within the miRNA precursor was identified that might be capable of modulating the expression and processing of this miRNA (SNP: rs11231899, rs11231898, rs15800646, rs15800644,

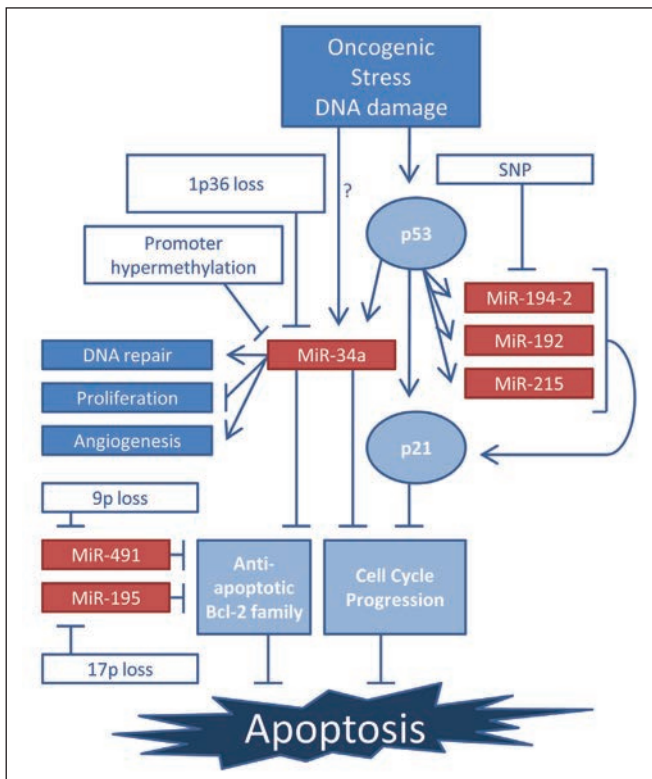


Figure 1. Regulation of apoptosis in CRC by miR-34a, miR-192, miR-194, miR-215, miR-195, and miR-491. Abbreviation: SNP = single nucleotide polymorphism

rs15800642, rs15800640, rs15800638).²⁴ SNPs have also been identified for miR-192 and miR-215 but these have not yet been associated with miRNA biogenesis. Copy number alterations of the genomic region of miR-192 (11q13.1), miR-194-2 (11q13.1), and miR-215 (1q41) have not been identified in CRC so far.

In vitro assays suggest the involvement of miR-195 and miR-491 in the evasion of apoptosis in CRC. Indeed, restoration of miR-195 expression in CRC cell lines reduced cell viability, promoted cell apoptosis *in vitro*, and suppressed tumorigenicity *in vivo*.²⁵ The pro-apoptotic function of miR-195 and miR-491 is mediated through inhibition of the translation of *BCL2* and *BCLXL* respectively, which are important anti-apoptotic molecules of the Bcl-2 family.²⁵ Transfection of miR-491 in CRC cell lines decreased Bcl-Xl protein expression and induced a 40% reduction in *BCLXL* mRNA levels. MiR-491 also induced a dose dependent decrease of cell viability and activates caspase 3/7 which are important apoptotic signalling molecules. Using a gain-of-function screen, miR-491 has also shown to be capable of suppressing proliferation.²⁶ Tumors derived from mice injected with miR-491-transfected CRC cells were around half the size of those derived from the negative control-transfected cells. Despite the fact that miR-491 inhibited cell proliferation of CRC cells *in vitro* and *in vivo*, there is no correlation between endogenous miR-491 expression and clinical outcome in human CRC.²⁶ MiR-491 is located at chromosome 9p21.3, and the 9p chromosome arm is a region that showed copy number loss in 16% of the CRC patients.²⁷ MiR-195 is down-regulated in CRC cell lines as well as in human CRC tissues²⁵ and is located at chromosome 17p13.1. Chromosomal losses are detected at chromosome 17p, a region that also contains the *TP53* tumor suppressor gene.²⁷ The functional relations of the different miRNAs in the escape of apoptotic cell death are summarized in Figure 1.

MIRNAS AND EPITHELIAL-MESENCHYMAL TRANSITION (EMT)

EMT is a cellular program converting polarized immotile epithelial cells into motile mesenchymal cells. This process enables cancer cells to promote their malignant phenotype and stem cell characteristics. Activation of EMT at the invasive front allows tumor cells to detach, migrate, and disseminate through blood or lymphatic vessels. Transforming growth factor beta (*TGFB1*), an EMT activator, is produced by tumor cells and triggers the expression of zinc-finger-enhancer binding protein 1/2 (*ZEB 1/2*). *ZEB1* represses E-cadherin transcription, promotes vimentin transcripton²⁸, and also directly suppresses transcription of the highly conserved miR-200 family (miR-141, miR-200b, and miR-200c), whose down-regulation is believed to be the essential feature of EMT. Surprisingly, one of the putative gene targets of the miR-200 family is

ZEB1 itself. Increased expression of miR-141 and miR-200c after knockdown of *ZEB1* in CRC cells, induced an epithelial phenotype with an increased E-cadherin expression and cell-cell adhesion, as well as reduced cell migration and invasion. Overexpression of miR-200c leads to translational inhibition of *ZEB1*, which induces mesenchymal-epithelial transition (MET) in cells that had previously undergone EMT.²⁹ This EMT-enhancing feed-forward loop of *ZEB1* and the miR-200 family is important in invading cancer cells and might explain the strong phenotypic heterogeneity often seen within individual tumors and metastases.³⁰ Depending on the initial signal, this loop could stabilize either mesenchymal or epithelial differentiation. The miR-200 family members are located in two clusters; miR-141 and miR-200c are located at chromosome 12p13.31 and miR-200b is located at chromosome 1p36.33. Expression of the miR-200c/141 cluster is regulated by DNA hypermethylation, demonstrating epigenetic regulation as a mechanism involved in the regulation of this miRNA locus.³¹ As mentioned before, loss of 1p36 is a recurrent aberration in CRC, indicating that there are two distinct mechanisms that regulate the expression of the miR-200 family.

Up-regulation of the EMT activator *TGFB1* also increases the expression of miR-21 and miR-31. Overexpression of these miRNAs *in vitro* facilitates and accelerates *TGFB1*-induced EMT, resulting in a higher percentage of cells adopting a 'spreading' morphology.³² Remarkably, the genomic location of miR-31, chromosome 9p21.3, show copy number losses in 16% of 45 CRC samples.²⁷ Other regulatory pathways for miR-31 are unknown. MiR-21 is located at 17q23.2, a genomic region that showed copy number gain in CRC. These copy number gains are more frequently observed in metastatic tumors, suggesting an important role for miR-21 in the metastatic pathway of CRC.²⁷ Regulation of EMT by miRNAs is depicted in Figure 2.

MIRNAS AND ANGIOGENESIS

Angiogenesis is essential for tumor growth and an important component in the metastatic pathway.³³ Tumor neovascularization is partly driven by hypoxia, which stimulates tumor cell production of angiogenic factors such as vascular endothelial growth factor-A (*VEGFA*). An important regulator involved in the cellular response upon hypoxia is *TP53*. As mentioned before, mutations in *TP53* are common in CRC¹⁶ and associated with increased tumor angiogenesis.³⁴ MiR-107 was identified as a downstream target of *TP53* and proved capable of inhibiting the translation of hypoxia inducible factor-1 beta (*HIF1B*).³⁵ Upon hypoxic signalling in a CRC cell line, overexpression of miR-107 resulted in decreased VEGF expression. In a mouse model, miR-107 overexpression resulted in decreased vascularity, lower VEGF expression, and smaller tumors. A loss of copy number of chromosome 10q, the genomic

location of miR-107, was observed in 11% of CRC patients.²⁷ In pancreatic cells, miR-107 was found to be epigenetically inactivated by promoter methylation but these findings have not been confirmed in CRC.³⁶

The oncogene *c-Myc* (*MYC*) is an important regulator of tumor angiogenesis. *MYC* is often co-activated with *RAS*, of which *HRAS* and *KRAS* are known to up-regulate *VEGF*.³⁷ In a CRC model the combination of mutations in *KRAS* and *TP53* yielded indolent, poorly vascularized tumors.³⁸ A robust tumor vasculature and progressive neoplastic growth only developed after overexpression of the *MYC* oncogene.³⁸ *MYC*-induced up-regulation of the miR-17-92 cluster is directly responsible for activating angiogenesis by down-regulation of the anti-angiogenic thrombospondin-1 (*THBS1*).³⁸ MiR-17-92 transduced cells formed larger, better-perfused tumors in mice models. The miR-17-92 cluster is located at 13q31³⁹, a genomic locus that is frequently amplified in CRC samples.^{27, 40} In Figure 3, the relationships of miRNAs with angiogenesis are depicted schematically.

MIRNAS AND INVASION, MIGRATION, AND PROLIFERATION

In tumors with invasive properties, intercellular adhesion is reduced due to loss of E-cadherin and proteolytic degradation of extracellular matrix (ECM) components. In this process, embedded growth factors and chemokines are liberated, thereby activating latent proteins on the cell surface. Binding of the epidermal growth factor (EGF) to the cell surface receptor (EGFR) induced stimulation of intracellular signalling pathways such as the RAS-RAF-MAPK and the PI3K-AKT pathways, resulting in increased invasion, motility, and proliferation. MiRNAs responsible for a more invasive phenotype can be divided into miRNAs acting in an EGFR-dependent manner and in an EGFR-independent manner.

RAS-RAF-MEK-MAPK pathway

Activation of the RAS-RAF-MAPK pathway component *PDCD4* directly suppresses invasion by the inhibition of the urokinase receptor (u-PAR) and the subsequent plasmin-mediated degradation of ECM components such as fibrin and collagen IV.⁴¹⁻⁴³ Overexpression of *PDCD4* in human CRC cells inhibited *AP1*-dependent transcription and subsequent invasion of CRC cells into Matrigel. Furthermore, down-regulation of *PDCD4* is associated with poor prognosis in resected CRC.⁴⁴ MiR-21 has a full match target sequence for *PDCD4*⁴⁵ and in both CRC cell lines and human CRC tissues, *PDCD4* is negatively regulated by miR-21 at the post-transcriptional level.⁴⁶ Overexpression of miR-21 induced invasion, intravasation, and metastasis in

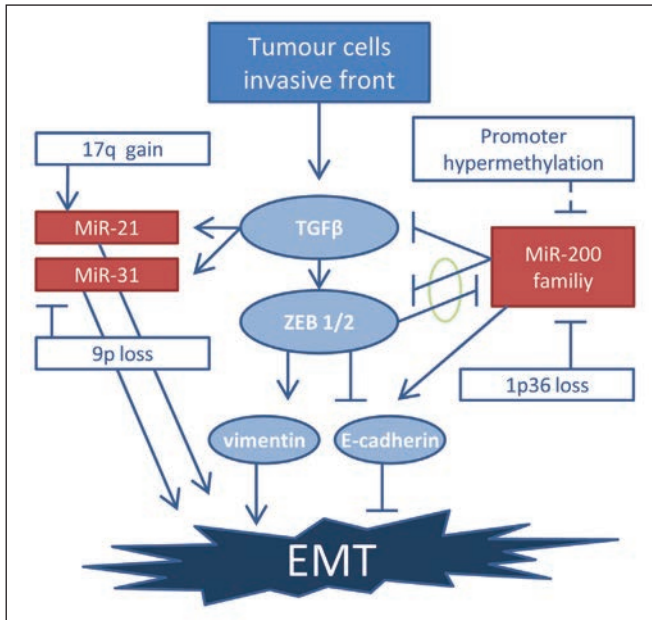


Figure 2. Regulation of epithelial-mesenchymal transition in CRC by miR-21, miR-31 and the miR-200 family. Abbreviations: TGFβ1 = transforming growth factor beta; ZEB1/2 = zinc-finger-enhancer binding protein 1/2; EMT = epithelial-mesenchymal transition; MET = mesenchymal-epithelial

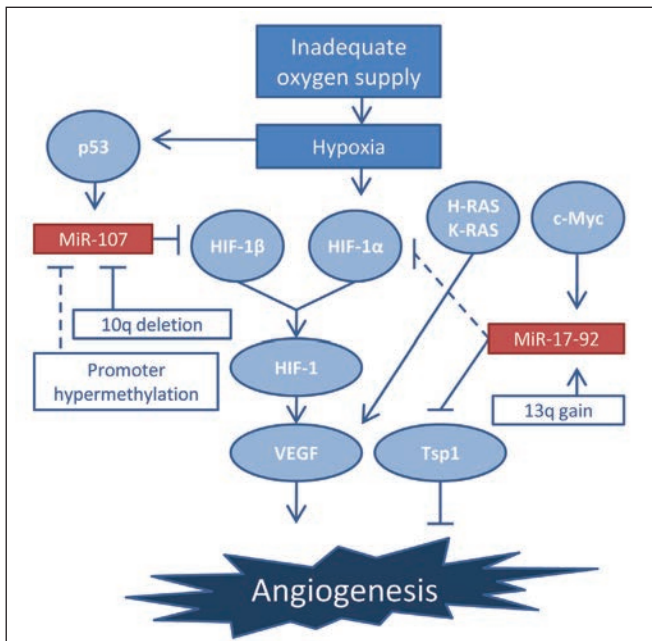


Figure 3. Regulation of angiogenesis in CRC by miR-107 and the miR-17-92 cluster. Abbreviations: HIF1 = hypoxia inducible factor-1; VEGF = vascular endothelial growth factor; THBS1 = thrombospondin-1

CRC cells.⁴⁶ This was confirmed *in vivo*, where miR-21 levels were positively correlated with the development of metastasis in human CRC.⁴⁷ MiR-21 is the most commonly and highly up-regulated miRNA in CRC, and besides *PDCD4*, multiple other gene targets of miR-21 have been recently identified. Sprouty (*SPRY2*), an inhibitor of fibroblast growth factor (FGF) signalling, branching morphogenesis, and neurite outgrowth, is one of the direct targets of miR-21. High levels of miR-21 in CRC cells lead to down-regulation of *SPRY2* and subsequent up-regulation of microvillus-like protrusions, resulting in a higher cell migration count.⁴⁸ Another target of miR-21 is tumor suppressor tropomyosin1, encoded by *TPM1*, an actin-binding protein stabilizing microfilaments which stimulate anchorage-independent growth in case of low expression.⁴⁹ Also, maspin⁵⁰, *PTEN*⁵¹, RECK, and TIMP3⁵² are regulated by miR-21 and involved in migration and the invasive potential of cancer cells. The genomic location of miR-21 (17q23.2) showed copy number gain in 16% of CRC patients.

In addition to miR-21, miR-31 positively regulates migration and invasion properties in CRC cells. T lymphoma invasion and metastasis 1 (*TIAM1*), a possible target for both miR-21 and miR-31, encodes a guanine nucleotide exchange factor of RAC regulating cell migration, invasion, and tumor progression. Repression of *TIAM1* is a critical component in miR-21/miR-31, stimulating the migratory and invasive properties of CRC *in vitro*.⁵³ However, the effect of these miRNAs *in vivo* is still unknown. Copy number losses of miR-31 (9p21.3) were infrequently observed in CRC patients.²⁷

The let-7 miRNA family and miR-143 are both capable of inhibiting *KRAS* translation, thereby blocking subsequent phosphorylation of MAPK and inhibiting growth. Both miRNAs act as a suppressor of colorectal tumorigenesis, but have not been directly linked to metastasis.⁵⁴ MiR-143 is located at chromosome 5q32, and CRC samples showed frequent deletions of 5q14-32.²⁷ Recently, a SNP (rs61764370) was observed in the let-7 miRNA complementary site of the 3' UTR of *KRAS* in non-small cell lung cancer (NSCLS). This SNP disrupted the let-7 regulation of the *KRAS* oncogene, resulting in an increased expression of *KRAS in vitro*.⁵⁵ Also, in metastatic CRC, this SNP was observed and related to tumor response in *KRAS* wild-type patients treated with cetuximab monotherapy.⁵⁶

In a human CRC cell line expressing a high level of endogenous miR-373, tumor cells depend upon this miRNA for their capacity to migrate in a trans-well cell migration assay.⁵⁷ In germ-cell tumors, miR-373 has the ability to suppress the oncogene-induced p53 pathway and cooperates with oncogenic *RAS* to promote cellular transformation through direct inhibition of the tumor suppressor *LATS2*.⁵⁸ Another target gene of miR-373 is *CD44*, which is a cell surface adhesion molecule, previously identified as a metastasis suppressor in CRC and capable of reducing the growth of experimental liver metastases.⁵⁹ MiR-373 expressing cells showed a reduction of the *CD44* gene product. In the human genome, miR-373 is located at chromosome 19q13.42. Copy number gains of chromosome 19q were observed in CRC patients.²⁷

The PI3K-AKT pathway

Receptor tyrosine kinase activation, mediated by the PI3K-AKT pathway, activates a cascade of anti-apoptotic and pro-survival signals.⁶⁰ *P85B*, a fundamental component of the PI3K signalling network, was identified as a candidate target gene of miR-126. Targeted degradation of p85 β by miR-126 impaired the downstream signalling cascade through which PIP3, PDK1, and AKT eventually induce cell proliferation, survival, and increased motility. MiR-126 is often down-regulated in CRC cell lines. Restoring miR-126 expression showed a dose-dependent reduction in cell and colony numbers.⁶¹ In breast cancer cell lines, restoration of miR-126 expression specifically decreased the lung colonizing activity and bone metastasis formation⁶², but this has not been confirmed in CRC. MiR-126 is located at chromosome 9q34.3, a region that is not observed to be deleted or amplified in metastatic CRC.

High level expression of miR-196a was observed in 75% of pancreatic adenocarcinomas, which was a negative predictive factor for survival.⁶³ MiR-196a-transfected CRC cells showed a significant increase in the growth of pulmonary metastases.⁶⁴ Increased phosphorylation of AKT was observed in miR-196a-transfected CRC cells, so induction of the pro-migratory phenotype is most likely linked to activation of the PI3K-AKT-mTOR pathway.⁶⁴ Moreover, miR-196a was significantly up-regulated in metastatic cancer cell lines, in contrast to cells isolated from the primary CRC. Two isoforms of miR-196a are known: miR-196a-1 is located at 17q21.32 and miR-196a-2 at 12q13.13. Copy number gains of chromosome 17q are observed in CRC, but there is no data available about the regulation of miR-196a-2 in human CRC.

EGFR-independent pathway

As mentioned before, decreased cell-cell adhesion and the recruitment of proteases to degrade the ECM provide tumor cells invasive properties independent of the EGFR pathway. Macrophage migration inhibitory factor (*MIF*) is a potential target of miR-451. This recently discovered oncogene is overexpressed in several human cancers and correlated with tumor aggressiveness and metastatic potential.⁶⁵ MiR-451 is often down-regulated in CRC, and these patients showed a significantly worse prognosis compared with those with overexpression.⁶⁵ Transfecting CRC cell lines with miR-451 significantly reduced the number of metabolically active cells as well as decreased proliferation. This miRNA is essential for the translocation of β 1 integrin to the basolateral membrane, which contributes to the formation of basolateral polarity in epithelial cells.⁶⁶ MiR-451 is located at chromosome 17q, a genomic region showing copy number gains in CRC patients. As mentioned before, metastasizing tumors tend to have more gains of 17q compared with non-metastasizing tumors.

Previous studies have linked up-regulation of metastatic tumor antigen 1 (*MTA1*) to the maintenance and progression of invasive phenotypes in CRC. MTA1 is a component of the chromatin remodelling complex, modulating transcription of its target chromatin by recruiting

histone deacetylases (HDACs) or RNA Polymerase II⁶⁷, and is regulated by miR-661.⁶⁸ Member of c/EBP α , a family of transcription factors, interact directly with the miR-661 promoter and positively regulate miR-661 expression. Expression of miR-661 and its activator c/EBP α are progressively reduced during cancer progression in breast cancer cell lines. Loss of miR-661 allowed *MTA1* levels to be sustained⁶⁸, and introduction of miR-661 inhibited the motility, invasiveness, and anchorage-independent growth of invasive breast cancer cells. Although up-regulation of *MTA1* was observed in CRC, a correlation between miR-661 and *MTA1* was only experimentally observed in breast and prostate cancer cells. In the human genome, miR-661 is located at chromosome 8q24.3. High-level copy number gains were observed at chromosome 8q, a region that also maps the *MYC* oncogene.²⁷ In CRC, the chromosomal regions containing miR-451 (17q) and miR-661 (8q) are often amplified, which is conflicting with the described anti-oncogenic function of these miRNAs *in vitro*. These contradictorily results might be explained by other mechanisms for miRNA dysregulation. The effect of chromosomal amplification of a miRNA gene could be perished by DNA promoter hypermethylation or decreased expression of components of the miRNA machinery. We cannot exclude combinatorial dysregulation of miR-451 and miR-661, but currently evidence is lacking. Figure 4 gives a schematic overview of the different miRNAs related to regulation of invasion, migration, and proliferation.

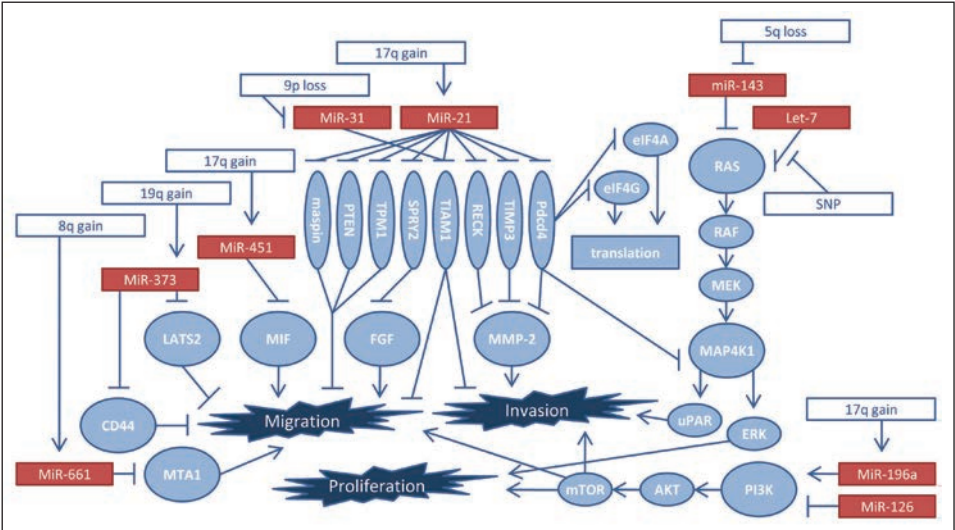


Figure 4. Regulation of invasion, proliferation, and migration in CRC by miR-21, miR-31, miR-143, Let7, miR-196, miR-126, miR-661, miR-520c, miR-373, miR-338-3p, and miR-451. Abbreviations: MTA1 = metastatic tumor antigen 1; LATS2 = large tumor suppressor homolog 2; MIF = migration inhibitory factor; FGF = fibroblast growth factor

FUTURE PERSPECTIVES AND CLINICAL APPLICATION

Evidence is accumulating that miRNAs play an important role in cancer progression and metastasis. In breast⁶² and hepatocellular cancer⁶⁹, there are already miRNA signatures identified that could distinguish primary tumors with metastases from metastasis-free tumors. We have summarized many miRNAs and their known target genes associated with the metastatic process that are dysregulated in CRC (Table 1), but only limited data are available that link miRNA expression to the metastatic phenotype in CRC patients. To gain better insight into dysregulated miRNAs involved in CRC metastasis, one approach is to compare miRNA expression profiles in primary tumors and metastatic tissue. So far, only two studies compared these miRNA expression profiles, of which Kulda *et al.* used an unmatched-pair approach.⁷⁰ This type of unmatched comparison is unreliable, due to the differences in endogenous miRNA levels between primary colorectal carcinomas. In order to overcome this problem, comparison of matched metastatic and primary tumor tissue is essential. Baffa *et al.* compared miRNA microarray analyses of ten primary colon tumors with one of their related metastatic lymph nodes.⁷¹ This analysis identified 21 miRNAs that were differentially expressed between primary colon tumors and their related metastatic samples, of which four miRNAs were validated with qRT-PCR. Several significantly deregulated miRNAs observed in their analyses have already been reported as being related to processes of tumor invasion and metastasis. The use of microarray analysis in paired tissue is a useful approach for identifying candidate miRNAs involved in metastasis. Therefore, this approach should be applied to a large cohort of patients with CRC metastasis. A second approach is to compare miRNA expression in primary tumors of patients with and without metastatic disease, to identify changes in miRNA expression of primary tumors that are more likely to metastasize. MiRNA expression patterns of primary breast tumors were analyzed in patients with different clinical outcomes by comparing metastatic tumors with non-metastatic tumors. An aberrant miRNA expression signature was identified that distinguishes between metastatic and non-metastatic breast cancer.⁶² MiRNA analysis of the primary tumor might therefore be an important tool for predicting individual prognosis. In both approaches, the location of miRNA expression (stroma, tumor cells) should be investigated. This may be important as stromal cells can be a source of miRNA expression and influence tumor progression, survival, and patient outcome.

MiRNA expression is probably not only associated with the development of metastases and prognosis, but might be correlated with therapeutic outcome as well. High expression of miR-21 in colon tumors of patients receiving adjuvant fluorouracil-based chemotherapy has been correlated with poor therapeutic outcome in two cohorts.⁷² Validation of these findings should be performed using larger cohorts and stratified for different types of adjuvant chemotherapy. Despite the fact, nowadays, that targeted therapies (bevacizumab, cetuximab, panitumumab)

Table 1. All MiRNAs regulating target genes involved in CRC metastasis

MiRNA	Chromosomal location	Target gene	Putative function	Expression in CRC	Copy number alteration	Other regulatory mechanisms
Let-7 family	Multiple members (3,9,11,19,21, 22)	<i>KRAS</i>	Invasion	Down-regulated		SNP in <i>KRAS</i>
MiR-17-92	13q31	<i>THBS1</i>	Angiogenesis	Up-regulated	Gain	
MiR-21	17q23.2	<i>PDCD4, SPRY2, TPM1, maspin, PTEN, RECK, TIMP3, TIAM1</i>	EMT, invasion, migration	Up-regulated	Gain	
MiR-31	9p21.3	<i>TIAM1</i>	EMT, invasion, migration	Up-regulated	Loss	
MiR-34a	1p36.22	<i>BCL2, E2F</i>	Cell-cycle progression, proliferation, apoptosis, DNA repair, angiogenesis	Down-regulated	Loss	Promotor hypermethylation <i>TP53</i> inactivation
MiR-107	10q23.31	<i>HIF1B</i>	Angiogenesis		Loss	Promotor hypermethylation
MiR-126	9q34.3	<i>P85B</i>	Invasion	Down-regulated		
MiR-141	12p13.31	<i>TGFB1</i>	EMT	Down-regulated		Promotor hypermethylation
MiR-143	5q32	<i>KRAS</i>	Invasion	Down-regulated	Loss	
MiR-192	11q13.1	<i>P21</i>	Apoptosis	Down-regulated		<i>TP53</i> inactivation
MiR-194-2	11q13.1	<i>P21</i>	Apoptosis	Down-regulated		SNP in pre-miR-194-2 <i>TP53</i> inactivation
MiR-195	17p13.1	<i>BCL2</i>	Apoptosis	Down-regulated	Loss	
MiR-196a	17q21.32/ 12q13.13	<i>AKT</i>	Invasion, migration	Up-regulated	Gain 17q	
MiR-200b	1p36.33	<i>ZEB1</i>	EMT	Down-regulated	Loss	
MiR-200c	12p13.31	<i>ZEB1</i>	EMT	Down-regulated		Promotor hypermethylation
MiR-215	1q41	<i>P21</i>	Apoptosis	Down-regulated		<i>TP53</i> inactivation
MiR-373	19q13.42	<i>LATS2, CD44</i>	Invasion, migration	Up-regulated	Gain	
MiR-451	17q11.2	<i>MIF</i>	Invasion	Down-regulated	Gain	
MiR-491	9p21.3	<i>BCLXL</i>	Apoptosis, proliferation	Down-regulated	Loss	
MiR-661	8q24.3	<i>MTA1</i>	Invasion	Unknown	Gain	

Abbreviations: MiRNA = microRNA; CRC = colorectal cancer;
EMT = epithelial to mesenchymal transition

are part of the treatment strategy in metastatic CRC patients, there are no studies available evaluating the predictive value of miRNAs in treatment response to these therapies.

When specific miRNAs involved in the process of metastasis are identified, therapeutic strategies can be developed that aim at the silencing of oncogenes or up-regulating tumor suppressor genes. In order to silence an oncogene, reintroducing or over-expressing the miRNA that targets the oncogene is needed. Overexpression can be realized by introducing synthetic miRNA mimics or chemically modified oligonucleotides. This new approach is called 'miRNA replacement therapy'. Vidic *et al* investigated the induced expression of KRAS targeting miRNA molecules on KRAS expression and growth of a CRC cell line.⁷³ Inhibition of the *KRAS* oncogene resulted in growth inhibition *in vitro* and *in vivo*. This suggests that KRAS silencing could be used therapeutically in the treatment of different cancers.⁷³ Wiggins *et al.* demonstrated the effects of miRNA overexpression by using chemically synthesized miR-34a and a lipid-based delivery vehicle that blocks tumor growth. Intra-tumoral injections of miR-34a prevented the outgrowth of viable tumors in tumor-bearing mice, as well as reduced expression of ki67 and increased expression of caspase-3 in the few viable tumor cells. This indicated that miR-34a actively inhibits proliferation and stimulates the apoptotic cascade. In addition, protein levels of direct miR-34a targets were repressed in tumors that received the miR-34a mimic.

A second option for cancer treatment could be the down-regulation of miRNAs that suppress the function of tumor suppressor genes. This may be achieved by the introduction of antisense oligonucleotides or antagomirs, which are synthetic analogues of miRNAs that bind irreversibly to and inhibit the function of the miRNA of interest. An example is the stable expression of the adenovirus type *E1A* gene in cancer cells that can reduce their tumorigenic potential and promote apoptosis through down-regulation of miR-520h. In addition to the tumor-suppressive activity, expression of the *E1A* gene was able to convert human cancer cell lines from the mesenchymal phenotype into an epithelial-like phenotype, thereby inhibiting metastasis.⁷⁴ These promising results can provide a new opportunity to inhibit cancer metastasis.

CONCLUSION

MiRNAs are important regulators of gene expression, and current data suggest an important role for miRNAs in the process of cancer metastasis. The genetic and epigenetic alterations that provide cancer cells the ability to metastasize are complex, and only a limited number of experimental models are available that allow examination of this in detail. In comparison with other tumor types such as breast cancer, experimental data on the role of miRNAs in CRC metastases are still limited. However, several candidate miRNAs have been identified that are promising targets for therapeutic interventions. Most of the studies identifying these candidate miRNAs are based on a small number of patients. It is inevitable that there are more unidentified miRNAs involved in the metastatic pathway of CRC. Therefore, we recommend identification of candidate miRNAs in a large cohort of metastatic CRC patients by comparing miRNA expression in metastatic and primary tumor tissue. Another approach to select miRNAs involved in metastasis is to compare miRNA expression in primary tumors of patients with and without metastatic disease. The next step is to gain insight into the biological consequences of these miRNAs on genes responsible for metastasis, using *in vivo* human CRC models. This useful approach should designate candidate miRNAs for further use as therapeutic targets in CRC treatment.

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Chapter 8 Chromosomal copy number aberrations in colorectal metastases resemble their primary counterparts and differences are typically non-recurrent

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SUBMITTED

ABSTRACT

The metastatic process is complex and remains a major obstacle in the management of colorectal cancer (CRC). To gain a better insight into the pathology of metastasis, we investigated genomic aberrations in a large cohort of matched CRC primaries and distant metastases from various sites. In total, 62 primary colorectal cancers, and 68 matched metastases (22 liver, 11 lung, 12 ovary, 12 omentum, and 11 distant lymph nodes) were analyzed by high resolution array comparative genomic hybridization for DNA copy number aberrations. Findings were validated using a publicly available dataset consisting of 21 primary tumors and matched liver metastases. Fluorescence in situ hybridization (FISH) was used to confirm some of the DNA copy number aberrations observed. Cluster analysis showed that metastases were highly correlated to their matched primary tumors in the majority of the patients. Recurring differences in chromosomal copy number aberrations between metastases and primaries were not observed in more than 2 of the 62 patients. Our data indicate that chromosomal copy number aberrations in colorectal metastases resemble their primary counterparts, and differences are typically non-recurrent.

INTRODUCTION

Metastatic disease is the principal event leading to death in patients with colorectal cancer (CRC), yet our understanding of the molecular events leading to metastasis is still incomplete. Tumor progression towards metastasis, meaning that malignant cells spread from the primary tumor to colonize distant organs, involves multiple steps, including invasion, intravasation, survival in the circulation, extravasation and colonization of the distant parenchyma.^{1,2}

A variety of genetic and epigenetic events that lead to loss of function of tumor suppressor genes, such as *APC*, *TP53* and *SMAD4* and gain of function of oncogenes like *KRAS* and *MYC*, drive tumor cell behavior in a Darwinian selection process. Two hypotheses aim to explain how tumor cells acquire the (epi)genetic alterations that make them proficient to metastasize. The “traditional model” suggests that the metastatic process is accompanied by a sequential accumulation of (epi)genetic alterations.³ Tumor cells pass through successive rounds of clonal progression and the most malignant cancer cells acquire the capacity to seed new colonies at distant sites.⁴ An alternative “predestination” hypothesis, implies that the capacity to metastasize is largely determined by the mutant alleles that are acquired relatively early during tumorigenesis.⁵ Subsets of genetic aberrations responsible for oncogenic transformation are also involved in the metastatic progression. This model does not question clonal selection or the accumulation of genetic alterations, but does not place metastatic dissemination near the end of tumor progression.⁶ According to this model, primary tumors that can and cannot metastasize will differ more in their biologic features than primary tumors and their associated metastases.

A better understanding of the biology behind the metastatic process can have a major clinical impact in personalized cancer treatment for several reasons. First, if the metastatic potential is already encoded in the bulk of primary tumors, it could be of prognostic value for predicting metastatic recurrence of CRC patients. Second, genetic profiling of relevant pathways could predict the response to treatment. Especially in the era of targeted therapy it is important to understand the differences and commonalities in the genetic make-up of primaries and their matched metastases in order to determine which tumor tissue best reflects the presence of targets for therapy. Third, if specific molecular alterations responsible for metastasis do exist, characterization of these could eventually lead to the development of new anti-metastatic therapies.

So far, most studies aimed to unravel metastasis-associated genomic alterations by comparing the genetic profile of metastases with unmatched primary tumors.^{7,8} This approach is of limited value due to the heterogeneity between individuals in the genetic profile of their tumors. There are well designed tissue based studies using ‘matched’ primaries and metastasis.^{9,10} These studies revealed that gene expression and copy number patterns of metastatic tumor cells

were strikingly similar to that of the primary tumor. Recurrences reported were not independently confirmed in larger datasets using independent techniques. Since the publication by Stange et al.⁹ the array comparative genomic hybridization (array CGH) technique has dramatically improved. The oligo array CGH technique used here allows for a 20-fold higher spatial detection resolution, with also the capability of detecting focal aberrations.¹¹⁻¹⁵

In order to improve our understanding of the biology behind the metastatic process, we conducted such high resolution array CGH analysis on a large set of primary CRC and matched metastases of various distant sites.

MATERIALS AND METHODS

Patients and tumor samples

Formalin-fixed paraffin-embedded (FFPE) tissue of surgically resected primary tumor, matched distant metastasis and matched normal colon, was obtained from 62 patients. For 6 patients, tissue samples of two different metastatic sites were collected. The 68 metastatic tissue specimens consisted of 22 liver metastases, 11 lung metastases, 12 ovarian metastases, 12 omental metastases, and 11 distant lymph node metastases. Eighteen patients included in this analysis participated in the CAIRO study¹⁶ (CKTO 2002-07, Clinical Trials.gov; NCT00312000) and 36 patients were treated in the CAIRO2 study¹⁷ (CKTO 2005-02, ClinTrials.gov; NCT00208546) of the Dutch Colorectal Cancer Group (DCCG). Written informed consent required for all patients before study entry also included translational research on tumor tissue. FFPE tissue of another 8 patients was collected from the tissue archive of the Department of Pathology at the Radboud University Nijmegen Medical Centre.

Clinical and histopathological parameters

The following clinical features were collected for each patient: age, gender, site of the primary tumor, metachronous (> 6 months after initial diagnosis) or synchronous (> 6 months of initial diagnosis) onset of metastases. The TNM classification (5th ed.)¹⁸ was used to describe the extent of cancer spread in terms of invasion depth and lymph node stage. Tumors were histologically classified using the World Health Organization guidelines.¹⁹ A tumor was considered to be of the mucinous type when at least 50% of the tumor volume consisted of mucin. Primary tumors were graded into well, moderately and poorly differentiated adenocarcinomas based on the part of poorest differentiation in the tumor. The mismatch repair system (MMR)

status was determined by immunohistochemistry and microsatellite instability (MSI) analysis.²⁰ Clinical and pathological parameters are summarized in Table 1.

Table 1. Baseline characteristics of the 62 patients included in the analysis

		Patients n, (%) (n=62)
Gender	Male	33 (53%)
	Female	29 (47%)
Age	Median (range)	60 (34-77)
Site of primary tumor	Colon	29 (47%)
	Rectosigmoid	15 (24%)
	Rectum	16 (26%)
	Unknown	2 (3%)
Onset metastases	Metachronous	30 (48%)
	Synchronous	32 (52%)
Diameter	Median (range)	40 (15-135)
Invasion depth	T1-2	5 (8%)
	T3	47 (76%)
	T4	10 (16%)
Lymph node status	N0	12 (19%)
	N1	22 (35%)
	N2	26 (42%)
	Unknown	2 (3%)
Classification	Adenocarcinoma	54 (87%)
	Mucinous carcinoma	8 (13%)
Differentiation grade	Well	3 (5%)
	Moderate	35 (56%)
	Poor	24 (39%)
MSI status	dMMR	2 (3%)
	pMMR	60 (97%)
Site of metastases	Liver	22 (32%)
	Lung	11 (16%)
	Omental	12 (18%)
	Ovarian	12 (18%)
	Distant lymph node	11 (16%)

Chromosomal copy number detection by array CGH

The procedures for DNA isolation, labelling and hybridization have been described previously.²¹ Briefly, DNA was isolated from an area containing at least 70% tumor cells. DNA was labelled and hybridized to dual channel Agilent 4x180K array CGH (Gene Expression Omnibus (GEO) platform GPL8687 Agilent Technologies, Palo Alto, USA). These arrays contain 180.880 in-situ synthesized 60-mer oligonucleotides, representing 169.793 unique chromosomal locations distributed across the genome at 17 kb intervals and are enriched with 4548 additional unique oligonucleotides, located at 238 of the Cancer Census genes.

Array CGH preprocessing

Array image analysis was performed and local background was subtracted from the signal median intensities of both tumor and normal DNA. The \log_2 tumor to normal ratio was calculated in the statistical programming language R using the package CGHcall²² and was normalized against the median value of the \log_2 ratios of all the oligonucleotides mapped to the March 2006 human reference sequence (NCBI36/hg18) on chromosome 1-22 and X.

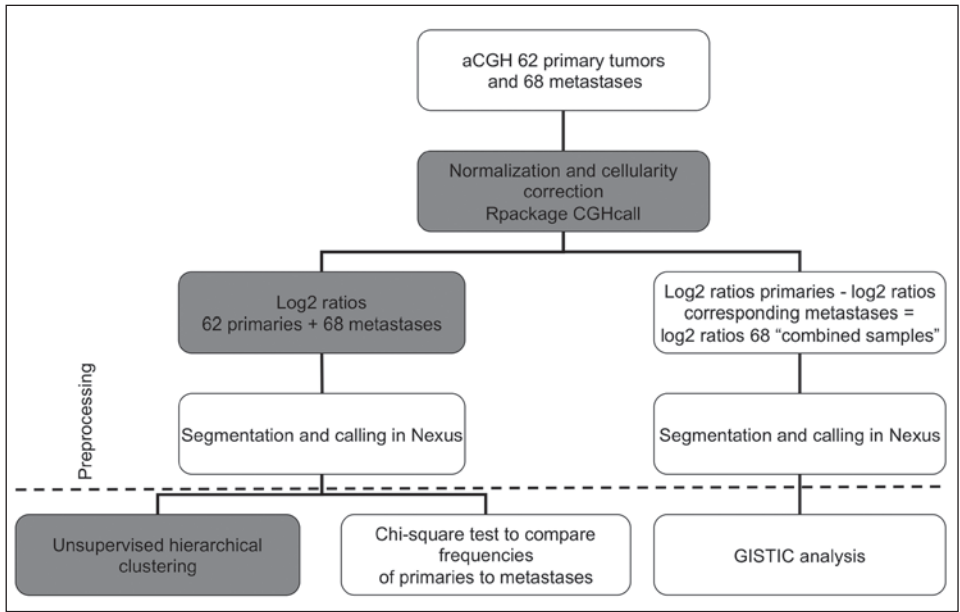


Figure 1. Flowchart of data preprocessing and analysis procedures. In grey analysis performed in R, in white analysis performed in Nexus.

The cellularity parameter in the CGHcall data analysis software was set according to the estimates made by the pathologist (I.D.N.). Further data interpretation and CNA calling was done with Nexus Copy Number 6.0 software (Biodiscovery, El Segundo, USA) using default settings, except for the Segmentation Algorithm, which was set to “Rank”. The CNA calling cut-off value for gene copy number gain or loss was set to 0.2 and -0.2, and for amplifications or homozygous deletions this cut-off value was set to 0.6 and -1.0, respectively (Figure 1).

Array CGH data analysis

Our dataset consisted of 62 primary tumor DNA copy number profiles and 68 metastatic profiles resulting in 130 profiles to analyze. Unsupervised hierarchical clustering analysis was performed in R with the segmented data according to the same procedures as Stange et al.⁹ The distance was calculated based on a Spearman correlation (Figure 1). For patients that did not cluster together, a likelihood of clonality was performed using the R package “clonality Bioinformatics”.²³ To compare frequencies of aberrant regions between metastases and primary tumors a chi-square test was performed within Nexus 6.0. This was done for all metastases as together and per metastatic site separately. Concordance of paired DNA copy number profiles was defined by the percentage of the genome with the same copy number in the metastasis and the corresponding primary tumor. To compare the samples in pairs (primary tumors and matched metastasis) DNA copy number ratios of the primary tumor were subtracted from the corresponding metastases, resulting in a dataset of 68 combined samples. The GISTIC algorithm²⁴ within Nexus 6.0 was used to identify genomic regions that are significantly amplified or deleted across this combined sample set. A gain or amplification means that higher DNA copy number ratios were detected in the metastasis compared to the primary tumor (Figure 1). For the regions that showed a deletion, lower DNA copy number ratios were detected in the metastases compared to the primary tumor. The output included those regions with a high corresponding G score, indicating either a high frequency of occurrence or a high amplitude for several samples or a combination of the two. The method accounts for multiple-hypothesis testing using the false-discovery rate (FDR), and a FDR below 0.05 was used as a level of significance. For validation purposes, the normalized array CGH data of Stange et al.⁹ including 21 primary tumors and matched liver metastases were re-analyzed in the same manner as described above.

Detection of chromosome 6q21 and 8q24.21 (MYC) co-amplification in large independent cohorts of primary colorectal tumors

The presence of the co-amplification of chromosome 6q21 and 8q24.21 (MYC) was assessed in array CGH data of 542 primary colorectal tumors. These array CGH profiles were

derived from 349 primary colorectal tumors who participated in either the CAIRO¹⁶ or the CAIRO2¹⁷ study (JC Haan et al., in preparation), and from 193 primary colorectal tumors present in The Cancer Genome Atlas (TCGA). Chromosomal amplifications were identified by CGHcall²² for the CAIRO and CAIRO2 samples, and by cBio Cancer Genomics Portal (<http://www.cbioportal.org>)²⁵ for the samples of the TCGA dataset, and were only acknowledged if the log₂ ratio of the segmented values was higher than 2.

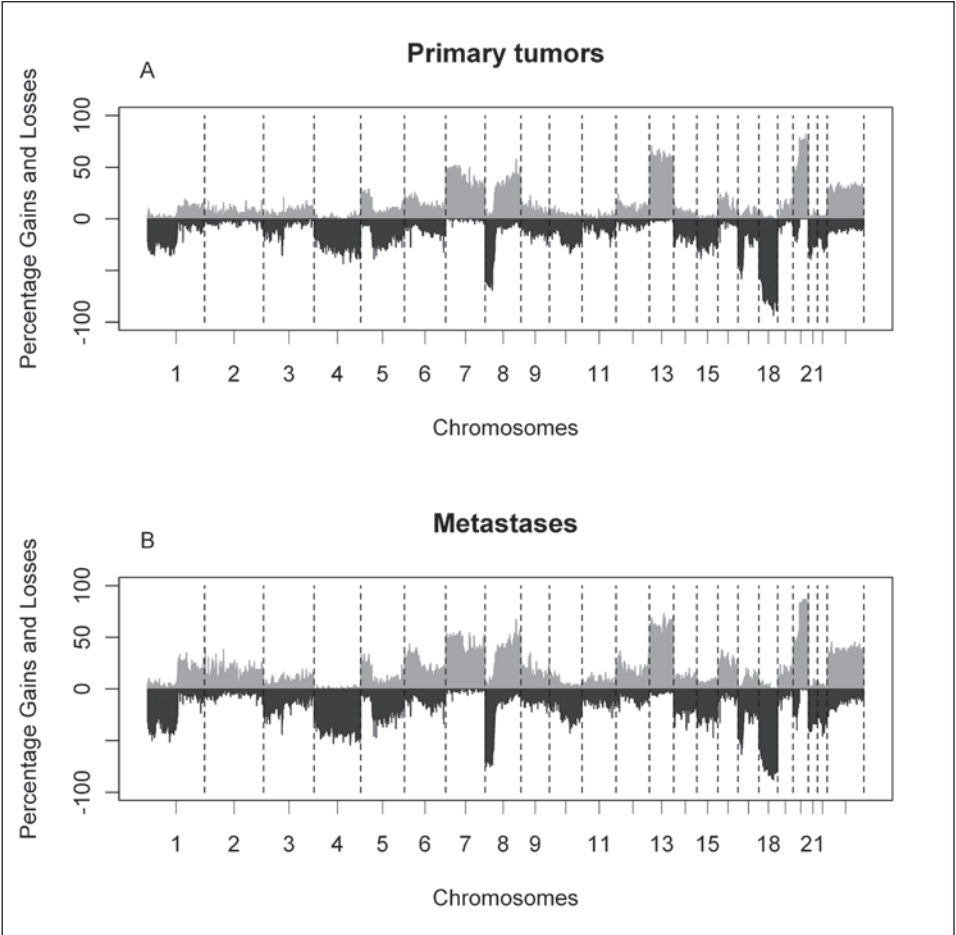


Figure 2. Frequency plots of DNA copy number aberrations in 62 primary tumors and 68 matched metastases. **(A)** Frequencies of aberrations based on called data for primary tumors and **(B)** metastases. The x-axis displays clones spotted on the array sorted by chromosomal position. The y-axis displays the frequency of tumors with gains (above zero) or losses (below zero). Boundaries of chromosomes are indicated by dotted lines.

Fluorescence in situ hybridization (FISH)

Copy number status of both chromosome 6q21 and 8q24.21 (*MYC*), as well as of the centromeres of chromosome 6 and 8 were assessed by FISH analysis. The *MYC* locus probe, 6q21 locus probe, and the centromere probes of chromosome 6 and 8 (Vysis, Abbott Molecular, Abbott Park, Illinois, USA) were used following a standard protocol. Briefly, paraffin-embedded tissue section (4 μ m) were deparaffinized, air-dried, and pretreated using a solution of MW Sodium Citrate pH 6.0 of HDP EDTA pH 9.0 (Klinipath, Duiven, The Netherlands). The slides were digested with Pepsin (200 U/ml; Sigma-Aldrich, St. Louis, USA) for 15 to 30 minutes at 37°C and subsequently fixed for 5 minutes in 1% formaldehyde/PBS (Merck Millipore, Massachusetts, USA). Thereafter, the slides were dehydrated using increasing graded ethanol series, air-dried, and finally 10 μ l of probe mix was applied to each tissue section. The slides, covered with a coverslip and sealed with rubber cement, were then incubated in a hybridizer (Dako, Heverlee, Belgium) for 10 minutes at 80°C and overnight at 37°C. The next day, the slides were washed for 5 minutes at 45°C, 3 minutes at 73°C, 5 minutes at room temperature using salted Sodium Citrate (Immunologic, Duiven, The Netherlands). After dehydration using increasing graded ethanol series and air-drying, the slides were counterstained by applying DAPI (Vector Laboratories inc. California, USA). Hybridization signals were visualized using a Leica microscope (Leica Microsystems Inc., Rijswijk, The Netherlands). Signals for each probe were counted in at least 40 cells per tumor sample. Samples with a ratio greater than 3 between *MYC* or 6q21 versus the centromere signals, in 10% of cells or more, were scored positive for amplification.

RESULTS

Striking similarity in DNA copy number status between primaries and matched metastases

We analyzed 62 primary tumors and 68 matched metastases for DNA copy number aberrations. Patterns of DNA copy number aberrations between primaries and metastases were highly similar for the majority of the patients (Figure 2), confirming clonality.

By comparing the group of primaries versus the group of metastases, only gain of chromosomes 2p25.3 and 2q21.3 were more frequently observed in metastases compared to primaries ($p < 0.001$; Supplemental Table 1). However, after correction for multiple testing no significant regions were left ($FDR > 0.05$).

Cluster analysis was used to assess similarities and differences in DNA copy numbers profiles between paired primary tumors and metastases relative to similarities and differences between

different tumors. Metastases were highly correlated to their matched primary tumors for 56 patients (correlation higher than 0.55), which included the two MSI tumors present in our dataset. For 6 patients, metastases and the corresponding primary tumors were not joined pair

Supplemental Table 1. Comparison of copy number aberrations between primaries and metastases

Region	Location	Copy number status	Frequency in metastasis (%)	Frequency in primary tumor (%)	P-value	FDR
chr2:2,609,073-2,646,266	q21.3	Gain	36.8	8.1	< 0.001	0.42
chr2:2,646,266-2,768,821	p25.3	Gain	38.2	8.1	< 0.001	0.42
chr2:2,786,596-3,216,115	p25.3	Gain	39.7	9.7	< 0.001	0.42
chr2:2,768,821-2,786,596	p25.3	Gain	38.2	9.7	< 0.001	0.45
chr2:3,216,115-3,233,076	p25.3	Gain	35.3	9.7	< 0.001	0.45
chr2:3,233,076-3,250,127	p25.3	Gain	35.3	8.1	< 0.001	0.45
chr2:3,250,127-3,281,460	p25.3	Gain	33.8	8.1	< 0.001	0.45
chr2:3,281,460-3,312,704	p25.3	Gain	32.4	6.5	< 0.001	0.45

Abbreviation: FDR = false discovery rate

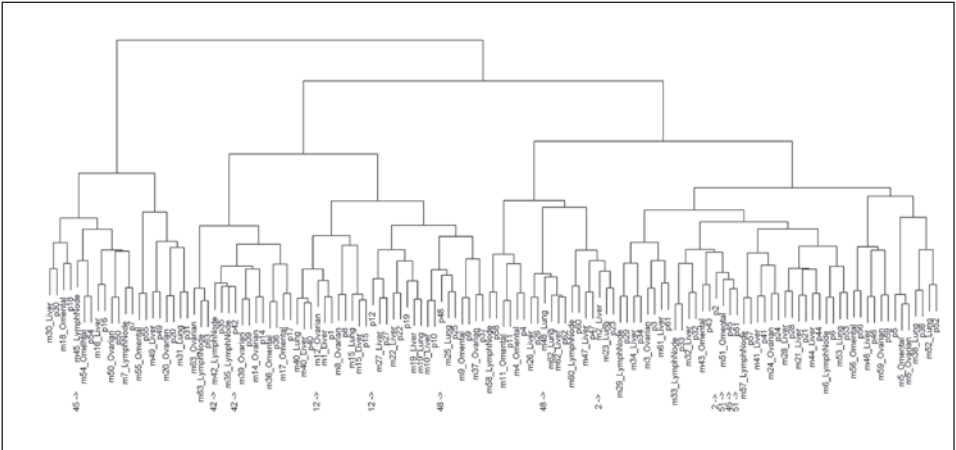


Figure 3. Dendrogram of unsupervised hierarchical clustering of DNA copy number aberrations of 62 primary CRC tumors (p) and 68 matched metastases (m). The numbers and pointers on the left show the patients of which the primary tumor and metastasis did not cluster together.

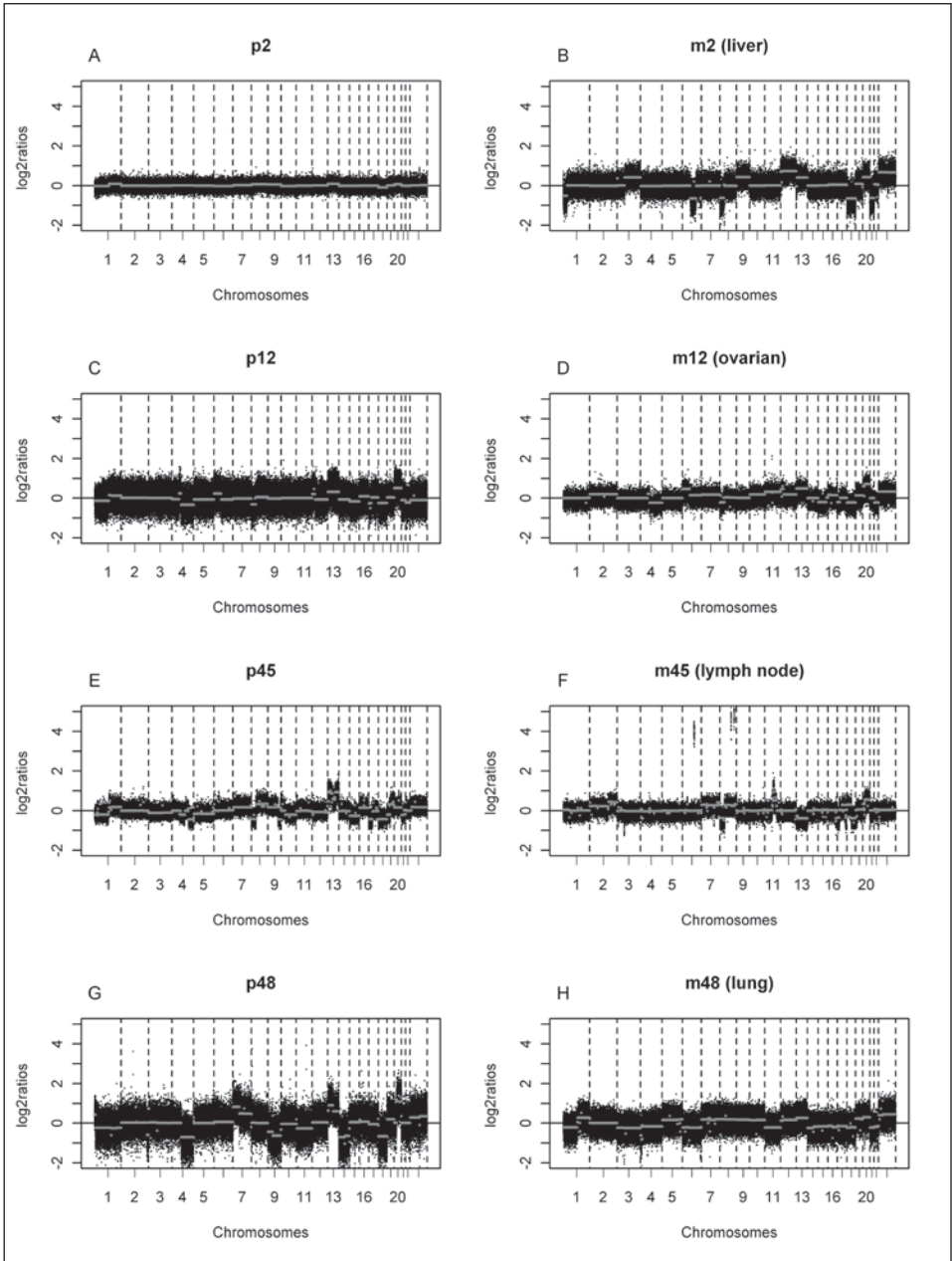
wise in the cluster dendrogram, with various distances (Figure 3). Histological re-evaluation of these 6 matched pairs showed similar morphologies. Two of the 6 patients however clustered with only one tumor or tumor pair between them. The remaining 4 copy number profiles are shown in Supplemental Figure 1. In these 4 patients we calculated the likelihood of clonality. The likelihood ratios of 3 of these samples were smaller than 1, statistically suggesting a low odds that the two tumors are clonal. For the other patient the likelihood ratio was 1.6 with a p-value of 0.042, statistically indicating that the tumor pair would be clonal.

Rare non-recurrent differences between metastases and primaries of individual patients

Even though a striking overlap of the aberration patterns was found between primary tumors and their matched metastases in the majority of the patients, also differences were observed. Overall, a median of 27% of aberrant genome was detected in primary tumors versus a median of 33% in the metastases. This difference is not a consequence of tumor cell percentage which was corrected for, nor a consequence of heterogeneity (data not shown). Pair wise comparison of the metastases and the primary tumor per patient revealed 4 patients with concordant segments for either gain, loss or normal DNA copy number of more than 95% (median 96.8% (95.6-100%)). In addition, 38 patients showed concordant segments between 70 and 95% (median 82.0% (72.0-94.7%)), and the remaining 20 patients had a concordance level in DNA copy number status of less than 70% (median 60.3% (41.6-69.7%)). There was no specific metastatic site overrepresented in one of these groups. Three of the four patients who did not join pair wise in the cluster analysis are in this last group with a concordance level of less than 70% (53.4, 67.8 and 69.7%). The remaining one who did not cluster had a concordance level of 82%. Representative examples of copy number profiles in each concordance level group are shown in Figure 4.

To establish which genomic regions show overall differences in copy number aberrations between the group of primary and metastatic tumors we generated a combined dataset. The \log_2 values of the primary tumors were subtracted from the \log_2 values of the metastases for each position (probe on the array) and analyzed these using the GISTIC algorithm in Nexus. GISTIC essentially combines ratios that are either highly recurrent or more sporadic, but with a high deflection. We observed 15 statistically significant events with, 13 regions of low DNA copy number ratios and 2 regions of high DNA copy number ratios (Supplemental Table 2). The significant peaks identified were each determined primarily by deflection rather than by frequency across the patient cohort, and most non-recurrent except for one co-amplification (see below).

We used the same approach of the combined data to assess the differences in copy number status between primary and metastatic tissue for each organ separately. The dataset of 22



Supplemental Figure 1. DNA copy number profiles of patients of which the correlation of the primary tumor (p) and their metastasis (m) was substantially low with more than one tumor pair between them. The patients showed concordant segments of (A-B) 67.8%, (C-D) 82.0%, (E-F) 69.7% and (G-H) 53.4%. The x-axis displays clones spotted on the array sorted by chromosomal position. The y-axis displays the \log_2 ratios of the clones. The segments are depicted by grey lines. Boundaries of chromosomes are indicated by dotted lines.

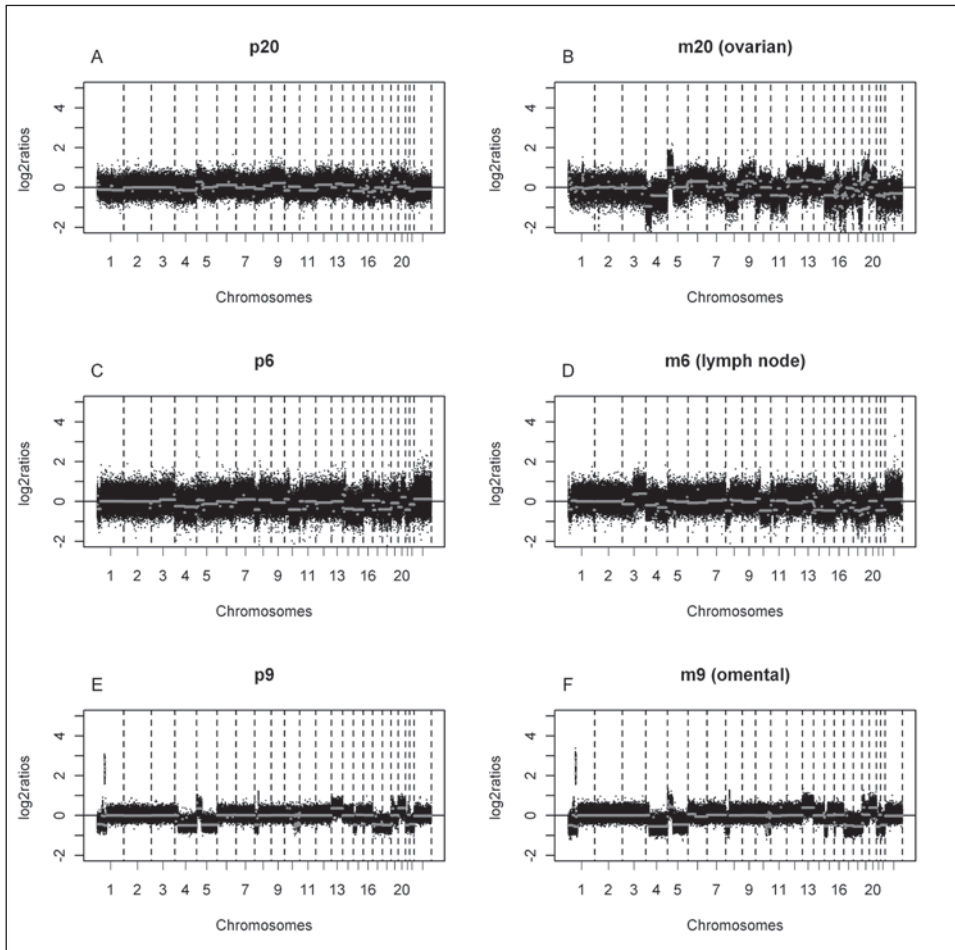


Figure 4. Representative examples of DNA copy number profiles of primary tumors (p) and their matched metastasis (m). **(A-B)** Example of a tumor metastasis pair from the group with less than 70% concordant segments (total 20 patients of which 3 patients were not joined pair wise in the dendrogram of Figure 3), **(C-D)** example of a tumor metastasis pair from the group with 70 and 95% concordant segments (38 patients of which 1 patient was not joined pair wise in the dendrogram of Figure 3), and **(E-F)** example of a tumor metastasis pair from the group with more than 95% concordant segments (4 patients). The x-axis displays clones spotted on the array sorted by chromosomal position. The y-axis displays the \log_2 ratios of the clones. The segments are depicted by grey lines. Boundaries of chromosomes are indicated by dotted lines.

patients with liver metastases revealed 7 regions with higher DNA copy number ratio and 8 regions with lower DNA copy number ratio in the liver metastases compared to the primary tumor. In the study used for validation (Stange et al.⁹) of patients with liver metastases, a difference between primaries and matched liver metastases was reported for chromosome 11p15.5 in 6 out of 21 patients. By re-analyzing the array CGH data of Stange et al.⁹ with the procedures we implemented, we confirmed the 11p15.5 gain in 6 liver metastases which was

Supplemental Table 2. GISTIC approach in combined samples

Extended Region	Type	FDR	G-Score	Genes
chr1:1,438,247-12,034,621	Loss	0.002	8.1	>50 genes
chr3:60,181,256-60,563,627	Loss	0.005	7.8	<i>FHIT</i>
chr4:4,626,306-31,693,271	Loss	0.05	6.8	>50 genes
chr4:85,226,936-92,648,432	Loss	0.006	7.7	>50 genes
chr6:105,350,190-107,821,131	Gain	< 0.001	12.6	<i>HACE1, LIN28B, BVES, C6orf112, POP3, POPDC3, PREP, PRDM1, ATG5, AIM1, RTN4IP1, QRSL1, AK124400, AK025967, LOC100422737, LOC553137, C6orf203, BEND3, PDSS2</i>
chr6:162,581,750-163,143,342	Loss	0.006	7.7	<i>Parkin, PARK2, PACRG</i>
chr8:39,380,297-39,805,805	Loss	< 0.001	13.4	<i>ADAM5P, tMDC, ADAM3A, tMDC III, ADAM18, ADAM2</i>
chr8:127,000,942-129,605,179	Gain	< 0.001	18.8	<i>LOC100130231, BX648371, FAM84B, AK125310, BC106081, DQ515898, DQ515899, DQ515897, POU5F1B, POU5F1, LOC727677, BC042052, MYC, MIR1204, TMEM75, PVT1, MIR1205, MIR1206, MIR1207, MIR1208, BC009730</i>
chr16:6,458,969-6,559,580	Loss	0.001	8.4	<i>A2BP1, RBFOX1</i>
chr18:3,458,748-13,680,560	Loss	< 0.001	8.8	>50 genes
chr18:48,738,234-57,604,348	Loss	< 0.001	10.8	>50 genes
chr20:14,932,222-15,035,264	Loss	< 0.001	9.9	<i>MACROD2</i>
chr21:35,205,722-35,651,123	Loss	0.003	8.0	<i>C21orf96, RUNX1</i>
chrX:7,022,874-7,471,041	Loss	< 0.001	11.2	<i>HDHD1A, HDHD1, MIR4767, STS</i>
chrX:88,378,677-88,554,287	Loss	0.002	8.2	No genes

Abbreviation: FDR = false discovery rate

absent in the corresponding primary tumors. This gain is based on BAC clones on their CGH arrays. In our set of 22 patients with liver metastases and the remaining 40 patients with other metastatic sites, gain of this region was not detected, despite the 38 oligonucleotides present on our arrays in the same chromosomal region.

In the 12 patients with omental metastases 6 regions showed significant peaks, of which 1 region showed a higher copy number ratio and 5 regions with a lower copy number ratio in the metastases compared to the primary tumor. In the other metastatic organs (ovary, lung, and distant lymph nodes) differences in DNA copy number ratios between metastases and primary

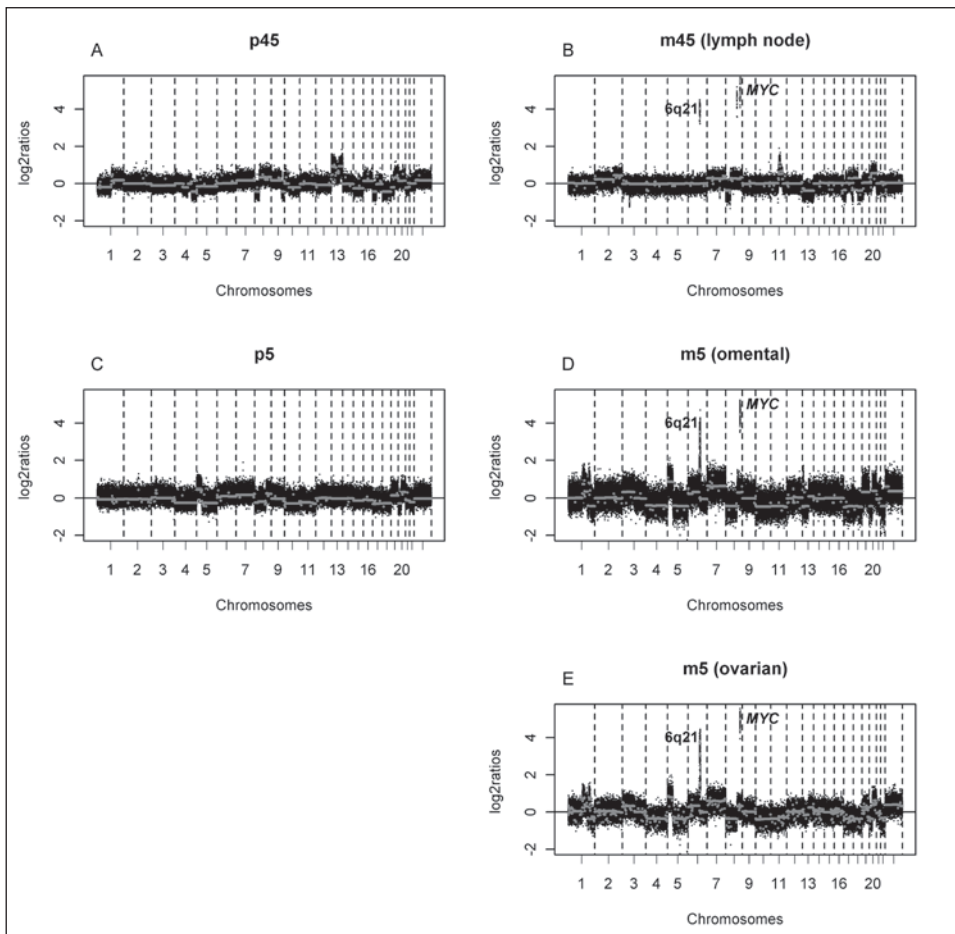


Figure 5. DNA copy number profiles of two patients containing a co-amplification of at 8q24.21 (MYC) and chromosome 6q21 in the metastasis (**B, D, E**), which was not present in the primary tumor (**A, C**). The x-axis displays clones spotted on the array sorted by chromosomal position. The y-axis displays the log₂ ratios of the clones. The segments are depicted by grey lines. Boundaries of chromosomes are indicated by dotted lines.

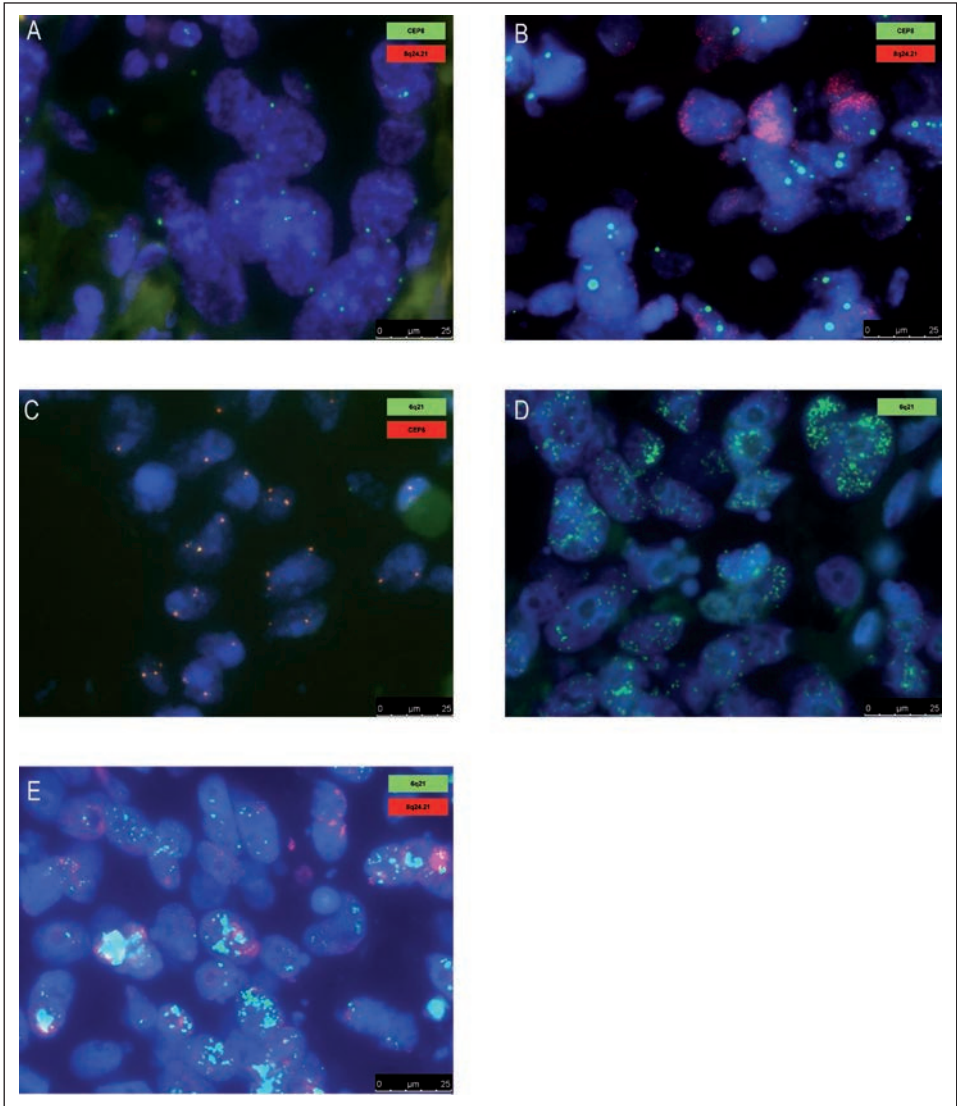


Figure 6. FISH analysis confirming high level co-amplification of *MYC* (8q24.21) and chromosome 6q21 in metastatic tissue (lymph node) which were absent in the matched primary tumor. (A) Primary tumor without the *MYC* amplification (red probe: *MYC* (8q24.21), green probe: centromere chromosome 8). (B) Distant lymph node metastasis with the *MYC* (8q24.21) amplification (red probe: *MYC* (8q24.21), green probe: centromere chromosome 8, gain). (C) Primary tumor without the chromosome 6q21 amplification (green probe: 6q21, red probe: centromere chromosome 6). (D) Distant lymph node metastasis with the 6q21 amplification (green probe: 6q21). (E) Co-amplification of *MYC* (8q24.21) and chromosome 6q21 in a distant lymph node (red probe: *MYC*, green probe: 6q21). Abbreviations: CEP6 = centromere chromosome 6; CEP8 = centromere chromosome 8.

tumors were not observed with any statistical significance. Analysis of the patients according to the different metastatic sites revealed no aberrant regions to be overrepresented in one of the metastatic subgroups.

Recurrent additional DNA copy number aberrations in metastasis

In only two patients we observed recurrent high level co-amplifications in the metastases, which were not detected by array CGH in the primary tumor. The amplifications were located at 6q21 and 8q24.21, the latter encompassing the *MYC* oncogene. One of these patients had two metastatic sites involved, both harboring this *MYC* amplification (Figure 5). All three *MYC* amplifications in the metastases had also chromosome 6q21 amplified. These high level amplifications of 6q21 also were not observed in the primary tumor. The array CGH results were confirmed by FISH analysis showing high level amplifications of *MYC* and chromosome 6q21 (Figure 6). The co-amplification did not result from translocation, since no co-localization of the amplified chromosomal regions was observed in the FISH analysis. We also performed FISH analysis to analyze heterogeneity within the primary tumor cell population. However, we did not observe subclones with high level amplification of *MYC* and/or chromosome 6q21. To confirm that the co-amplification is metastasis-specific we analyzed 349 primary colorectal tumors of the CAIRO studies and 193 primary colorectal tumors of the TCGA dataset. We detected high level amplification of *MYC* alone, once in a primary colorectal tumor of the CAIRO datasets and three times in the primaries of the TCGA dataset. The 6q21 amplification was only detected once in the CAIRO datasets, but not observed in the TCGA dataset. In none of these 542 primary tumors a co-amplification of *MYC* and chromosomal locus 6q21 was observed.

DISCUSSION

In a large set of clinical samples we observed highly similar DNA copy number aberrations in metastases compared to primary CRC in the majority of the patients. Copy number differences are present but rather than recurrent they were sporadic for individual patients.

Given the significant overlap in chromosomal aberrations between primary tumors and corresponding metastases in our dataset of 68 pairs, we validate the early findings by Stange et al.⁹ using 21 pairs. Therefore, we reason that many chromosomal aberrations arise in the primary tumor before metastatic spread. In the past years, genetic data have become available supporting the idea that the metastatic behavior seems to be predetermined relatively early in

tumorigenesis. First, micrometastases are observed in many individuals with small, low-stage tumors.²⁶ Second, RNA expression profiling of the bulk of primary tumors predict the metastatic recurrence of cancer patients.^{27,28} Third, microarray analysis revealed that RNA expression and DNA copy number patterns of metastatic tumor cells were strikingly similar to that of the primary tumor.^{9,10,29} Sequence analysis of coding regions in primary and metastatic tumor genomes also suggest that only a few mutations are required to transform cells from an invasive colorectal tumor into cells that have the capability to metastasize.³⁰ Genome wide sequencing of matched primary and metastatic tissues has only been performed in small patient cohorts. In the study of Kloosterman et al.³¹, significant overlap in somatic structural changes between 4 primary tumors and their corresponding metastases was observed. Moreover, whole-genome sequencing of matched primary pancreatic tumors and metastases³², and genomic analyses of primary prostate cancer and metastases³³ revealed highly similar genomic profiles in other solid malignancies as well. All these data suggest that essential mutations and chromosomal aberrations needed for cancer progression occur predominantly in the primary tumor before initiation of the metastatic spread, and are consistent with the genetic observations presented here.

Nevertheless, differences are observed in DNA copy numbers between primaries and metastases. There are several potential scenarios for the observed differences. Changes in chromosomal aberrations can occur because the primary and metastasis are different branches from a common yet heterogeneous ancestor³⁴, or *after* the dissemination of metastatic cells from the primary tumor. We hypothesize that the most likely scenario is a combination of both heterogeneity within the primary tumor and post-dissemination effects. It is thereby important to take into account that by array CGH chromosomal aberrations can go undetected if present in less than 30% of the tumor cells.³⁵ Since a primary colorectal tumor can be quite large and only a small cross section was taken for copy number analysis, heterogeneity would be reflected in the copy number measurements and explain some of the differences between matched primaries and metastases. Another explanation for the observed differences could be that some of the patients included in our cohort presented with metachronous metastases and consequently received (neo)adjuvant systemic treatment. The effect of systemic treatment on chromosomal instability however is largely unknown, but probably limited since in a small dataset no significantly increased number of gene variants (associated with pathways relevant in cancer) were observed as a result of chemotherapy.³⁶ Our data strengthen this observation because the patients who did receive (neo)adjuvant systemic treatment clustered pairwise. None of the 6 patients who did not cluster together received chemotherapy and targeted agents.

Stange et al.⁹ published a dataset of 21 paired samples where a characteristic gain was found in 1 out of 3.5 patients on chromosome band 11p15.5 in liver metastases. They report confirmation of this observation in an independent dataset of liver metastases (n=50). This obser-

vation could not be confirmed in our set of patients with liver metastases (n=22), nor in other metastatic sites and could not be detected in the Kloosterman dataset of matched primary metastasis pairs.³¹ In the cohort of patients studied here we observed only one recurrent event; two patients with co-amplifications on the same chromosomal locations in the metastases, which were not present in the primary tumor. This co-amplification was not detected previously in larger series of primary tumors, and smaller studies of metastases, including the TCGA CRC samples. These series of metastases did not use high resolution array CGH (not able to detect focal amplifications), so the association of this specific co-amplification with metastasis needs further confirmation in larger series using the current available high resolution array CGH. The relationship between 6q21 and 8q24.21 described in the literature is in hematologic malignancies where *PRDM1*, localized at chromosome 6q21, is able to decrease the expression of *MYC*.^{37,38} We hypothesize that these two amplifications would be obtained through selection and adaptation within developing metastases after dissemination.

In a previous array CGH analysis of our group, we observed more gains at chromosome 20p11 in primary tumors that metastasize to the liver compared to primary tumors that disseminate to extrahepatic organs.³⁹ We also observed more gains at chromosome 20p11 in liver metastases compared to the metastases of other distant sites (data not shown). However, comparison of the metastasis and primary tumor in each group separately (liver, lung, lymph node, omentum, and ovary), did not reveal any recurrent additional copy number aberrations within one group compared to the others. This also fits our hypothesis that the genetic program responsible for metastatic tropism is already acquired relatively early during tumorigenesis.

In the era of personalized anti-cancer treatment it is essential to know the diverging features between primaries and metastases. It is therefore important to determine which tumor tissue will best predict treatment outcome. Current clinical practice is to use archived material of the primary tumor to determine the constitution of the molecular target to select patients for treatment of the metastasis. If true additional genomic aberrations would be required for metastatic progression, these regions would be relevant to design adjuvant treatment strategies for stage II-III CRC. Our data showed that genomic profiles are highly similar, which makes it unlikely that precursor cells of overt metastases in CRC disseminate early to sites where they proceed to undergo their own divergent genetic evolution. Instead of focusing on additional genetic features responsible for metastatic formation, identification and understanding of the chromosomal aberrations in precursor cells of the primary tumor is probably more important and should be the route to the discovery of new drug targets.

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Chapter 9 Chromosome 20p11 gains are associated with liver- specific metastasis in patients with colorectal cancer

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ABSTRACT

OBJECTIVE: Metastatic colorectal cancer (CRC) cells have a selective preference for certain target organs that cannot be explained by circulatory patterns alone. This study aimed to identify clinicopathological features and chromosomal aberrations of primary tumors associated with organ-specific CRC metastasis.

DESIGN: Clinicopathological features were investigated in CRC patients with exclusively hepatic (n=182) versus exclusively extrahepatic (n=139) metastases. 139 primary tumors of patients with hepatic (n=85) and extrahepatic metastases (n=54) were screened for chromosomal aberrations by microarray-based comparative genomic hybridization, and the findings were validated in an independent set of 80 primary tumors. A publicly available database was used to correlate chromosomal aberrations with gene expression. Protein expression was evaluated by immunohistochemistry on tissue microarrays.

RESULTS: Patients with hepatic metastases were significantly more often male (71% vs 53% $p=0.002$), more often had abnormal lactate dehydrogenase activity (37% vs 14% $p<0.0001$), exhibited primary tumor localization in the colon (52% vs 40% $p=0.03$) and had synchronous onset of metastases (70% vs 19% $p<0.0001$). Primary tumors of patients with hepatic metastases were more commonly T3 tumors (79% vs 63% $p=0.006$) and less commonly of mucinous histology (5% vs 16% $p=0.02$). Gain of 20p11 was more often observed in patients with hepatic metastases ($p<0.05$), which was confirmed in an independent dataset ($p<0.05$; $FDR<0.05$). Twelve genes mapping at 20p11 were significantly overexpressed as a consequence of 20p11 copy number gain. *C20orf3* showed the strongest correlation between RNA expression and DNA copy number. This was reflected in significantly higher protein expression in patients with hepatic metastases (59%; $n=325$) than in those with extrahepatic metastases (41%; $n=256$) ($p=0.01$).

CONCLUSION: *C20orf3* mapping at 20p11 is associated with hepatic-specific metastasis in patients with CRC. This gene is a candidate biomarker for liver metastases and may be of clinical value in early-stage CRC.

INTRODUCTION

Colorectal cancer (CRC) is one of the most commonly diagnosed malignancies, and the majority of these patients die as a result of metastatic disease. The phenomenon of cancer metastasis has been extensively studied and characterized as a complex, multistep process. To produce metastatic outgrowth, tumor cells need to be proficient in all steps of the metastatic process, including invasion, embolization, survival in the circulation, arrest in a distant capillary bed, and proliferation within the organ parenchyma. Tumor cells acquire these biological properties by accumulating (epi)genetic alterations.¹ One hypothesis on the acquisition of a metastatic phenotype is that these modifications are already present in the primary tumor. This is supported by the finding that gene expression signatures of the primary tumor have been shown to predict the occurrence of metastasis in patients with breast cancer.²

Like other types of cancer, CRC shows organ preference for metastasis formation. The liver is the predominant site in approximately 80% of patients with CRC. In 40-50% of these patients, extrahepatic organs are also involved in metastatic colonization.³ Lung metastases develop in 5-15% of the patients, and metastases in the central nervous system, adrenal glands, ovaries, skeleton, and skin together account for <10% of all colorectal metastases.⁴ Metastatic cells prefer to grow in certain organs in a way that cannot be explained by circulatory patterns alone. Organ specificity has mainly been investigated in animal models,^{5,6} but gene expression profiling in human breast cancer tissue can predict bone and lung metastases.^{7,8} The development of DNA microarray technology, which allows for genome-wide profiling, has provided new insight into the genetic basis of metastasis. However, so far, neither chromosomal aberrations nor gene expression profiling in the primary tumor have been correlated with hepatic versus extrahepatic metastasis in CRC.

CRC in many aspects, including prognosis and survival, is a heterogeneous disease. In case of unresectable metastatic CRC, patients are treated with cytotoxic regimens (fluoropyrimidines, oxaliplatin, irinotecan) in combination with targeted therapy (vascular endothelial growth factor and epidermal growth factor receptor antibodies). There are conflicting data available on the prognostic value of organ-specific metastasis in patients treated with systemic therapy. Several studies have reported the presence of liver metastases as a negative predictor,⁹⁻¹⁴ while others observed survival benefit in patients with hepatic metastases compared with patients with lung metastases.¹⁵⁻¹⁷ So far, no studies have been performed to evaluate the differences in patients with hepatic versus extrahepatic metastases in terms of clinicopathological features and outcome.

The present study aimed to identify clinicopathological features, chromosomal aberrations and outcome associated with hepatic versus extrahepatic metastasis in patients with CRC.

MATERIALS AND METHODS

Patients

The patients included in this analysis participated in the CAIRO study (CKTO 2002-07, <http://ClinicalTrials.gov>; NCT00312000) of the Dutch Colorectal Cancer Group (DCCG).¹⁸ In this multicentre phase III trial, 820 patients with metastatic CRC without previous systemic treatment for metastatic disease were randomized between sequential and combination treatment with capecitabine, irinotecan and oxaliplatin. The primary end point of the study was overall survival (OS). The written informed consent required for all patients before study entry also included translational research on tumor tissue. For the present analysis, we selected 550 eligible patients who underwent a resection of the primary tumor, for which formalin-fixed paraffin-embedded (FFPE) material of the primary tumor was available. Patients were divided according to the site of the metastases in exclusively hepatic (n=182) and exclusively extra-hepatic (n=139) disease. Patients with a combination of hepatic and extrahepatic metastases (n=221), locally advanced disease (n=7), and for whom the metastatic site was unknown (n=1) were excluded from this analysis.

Clinical and histopathological parameters

The following clinical features were collected for each patient: age, gender, Eastern Cooperative Oncology Group (ECOG) performance status, serum lactate dehydrogenase (LDH), site of the primary tumor, previous adjuvant therapy, number of metastatic sites involved, metachronous (>6 months after initial diagnosis) or synchronous (\leq 6 months of initial diagnosis) onset of metastases, and regimen used as first-line treatment.

The TNM (tumor, node, metastases) classification was used to describe the extent of cancer spread in terms of invasion depth and lymph node stage.¹⁹ Histopathological review was carried out by two independent observers (LJMM, IDN). If the scoring was not unambiguous, the opinion of the pathologist (IDN) was final. Tumors were classified using the WHO guidelines.²⁰ A tumor was considered to be of the mucinous type when at least 50% of the tumor volume consisted of mucin. Primary tumors were graded into well, moderately and poorly differentiated adenocarcinomas based on the part of poorest differentiation in the tumor. The mismatch repair system status was determined by immunohistochemistry and microsatellite instability analysis.²¹

Data analysis of clinicopathological features and outcome

Clinical and histopathological characteristics of patients with hepatic and extrahepatic metastases were compared using the Wilcoxon signed rank test or χ^2 test where appropriate. OS was calculated as the interval from the date of randomization until death from any cause or until the date of last follow-up. Progression free survival (PFS) for first-line treatment was calculated from the date of randomization to the first observation of disease progression or death from any cause. OS and PFS curves were estimated using the Kaplan-Meier method and compared with the log-rank test. Patients were considered evaluable for response if they had completed at least three cycles of chemotherapy. Overall response was defined as partial response or complete response. Disease control was defined by stable disease with a duration of more than 4 months or partial response or complete response. Differences in response and disease control rates were analyzed by a χ^2 model. Multivariate analysis of OS was performed by means of a Cox proportional hazards model, including the following covariates: gender, performance status, serum LDH, site of the primary tumor, number of metastatic sites involved, T stage, N stage, classification, and differentiation grade of the primary tumor. All statistical tests were two-sided, and p values of <0.05 were considered significant.

Sample selection for DNA copy number profiling

To assess DNA copy number profiles we used an array comparative genomic hybridization (CGH) dataset of 222 primary colorectal tumors from patients with metastatic CRC who were treated within the CAIRO study (Haan *et al.* unpublished data). We selected the 85 and 54 CRC patients with exclusively hepatic and exclusively extrahepatic metastases, respectively. The accuracy of the observed differences in DNA copy number profile was validated in an independent validation set of 45 and 35 primary tumors with hepatic and extrahepatic metastases, respectively. These tumors were derived from an array CGH dataset of 134 metastatic CRC patients (Haan *et al.* unpublished data), who participated in the CAIRO2 study (CKTO 2005-02, <http://ClinicalTrials.gov>; NCT00208546).²² In this multicentre phase III trial, patients were randomly assigned to first-line treatment with capecitabine, oxaliplatin and bevacizumab, or the same schedule with the addition of cetuximab. Since the CAIRO2 study had a negative outcome, possibly because of a negative interaction between the study drugs, the array CGH profiles were determined only in patients receiving capecitabine, oxaliplatin and bevacizumab. The selection criteria for the patients used for both the training and validation set are described in detail elsewhere (Haan *et al.* unpublished data). Briefly, primary tumors were selected from patients who underwent a resection of the primary tumor and for whom FFPE material of the primary tumor was available. Since we used normal as well as tumor DNA from the same patient, FFPE material for both needed to be available. Stringent criteria were used to select patients based on tumor cell percentage (at least 70%), clinical

Supplemental Table 1. Clinicopathological features in the 85 patients with hepatic versus 54 patients with extrahepatic metastases used for DNA copy number profiling (CAIRO study; learning set)

		Hepatic metastases n = 85		Extrahepatic metastases n = 54		P-value
Age	Median (range)	66.0 (37.0-78.0)		63.5 (40.0-79.0)		0.87
Gender	Male	60	71%	31	57%	0.14
	Female	25	29%	23	43%	
WHO performance status	0-1	80	94%	53	98%	0.33
	2	5	6%	1	2%	
LDH at randomization	Normal	59	69%	48	89%	0.008
	> ULN	26	31%	3	11%	
Site of primary tumor	Colon	60	71%	56	60%	0.59
	Rectosigmoid	19	22%	49	28%	
	Rectum	5	6%	34	10%	
	2 sites	1	1%	1	2%	
Prior adjuvant therapy	No	78	92%	40	74%	0.007
	Yes	7	8%	14	26%	
Onset metastases	Metachronous	22	26%	39	74%	<0.0001
	Synchronous	63	74%	14	26%	
Metastatic sites involved	1	85	100%	28	54%	<0.0001
	2	0		18	35%	
	>2	0		6	11%	
Treatment arm	Sequential	45	53%	29	54%	0.93
	Combination	40	47%	25	46%	
Diameter	Median (range)	45 (20-130)		50 (15-100)		0.82
Invasion depth	T1-2	4	5%	5	9%	0.09
	T3	76	80%	34	63%	
	T4	13	15%	15	28%	
	Unknown	1		0		
Lymph node status	N0	29	36%	23	43%	0.65
	N1	29	36%	19	35%	
	N2	23	28%	12	22%	
	Unknown	4		0		
Classification	Adenocarcinoma	65	81%	36	69%	0.07
	Mucinous carcinoma	5	6%	11	21%	
	Other	15	13%	7	10%	
Differentiation grade	Well	1	1%	5	4%	0.08
	Moderate	51	65%	68	46%	
	Poor	27	34%	66	50%	
	Unknown	6		0		
MMR status	dMMR	3	4%	3	6%	0.57
	pMMR	82	96%	51	94%	

Abbreviations: WHO = World Health Organization; LDH = lactate dehydrogenase; ULN = upper limit of normal; MMR = mismatch repair; dMMR = deficient mismatch repair system; pMMR = proficient mismatch repair system

variables (matched to the stratification parameters in the original studies) and DNA quality (specific activity at least 16 pmol/μg). The clinicopathological features of patients in both the learning (Supplemental Table 1) and validation (Supplemental Table 2) array CGH datasets are representative of the larger dataset used for clinicopathological comparisons.

DNA isolation

DNA was isolated using an extensively validated protocol as previously described.²³ For each tumor, an area was marked containing at least 70% tumor cells. Of the FFPE blocks two to six 10 μm sections were cut, deparaffinized and microdissected. DNA was extracted using a column-based method (QIAmp microkit, Qiagen, Hilden, Germany). Matched normal mucosa DNA was used from all of these samples as a reference and was obtained from the resection margins or at least 1 cm distance from the tumor. Normality was confirmed *in silico* for each reference by comparing the array signals of the normal reference of another patient by across array.²⁴ All DNA concentrations were measured on a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA).

Array CGH

Labelling and hybridization was carried out as previously described.²⁴ Briefly, 500 ng genomic DNA and matched normal DNA were labelled using a CGH labelling kit for oligo arrays (Enzo Life Sciences, Farmingdale, New York, USA) with cyanine 3-dUTP and cyanine 5-dUTP nucleotide mixture, respectively. Labelled DNA quality was tested by using the Nanodrop ND-1000 spectrophotometer (Thermo Scientific) to measure specific activity. Samples with specific activity <16 pmol/μg were considered insufficient, and these cases were replaced by comparable samples. Hybridizations were performed on the Agilent 4x180K oligonucleotide arrays (AMADID number 022522, Agilent Technologies, Palo Alto, California, USA). These arrays contain 180880 *in-situ* synthesized 60-mer oligonucleotides, representing 169793 unique chromosomal locations distributed across the genome at 17 kb intervals and is enriched with 4548 additional unique oligonucleotides, located at 238 of the Cancer Census genes. The exact array design can be found in the Gene Expression Omnibus (GEO) platform GPL8687 <http://www.ncbi.nlm.nih.gov/geo/>. Images of the arrays were acquired using a microarray scanner G2505C (Agilent technologies) .

Preprocessing array CGH data and data analysis

Array image analysis was performed using Features Extraction software (V.10.5.1.1, Agilent Technologies). Local background was subtracted from the signal median intensities of both

Supplemental Table 2. Clinicopathological features in the 45 patients with hepatic versus 35 patients with extrahepatic metastases used for DNA copy number profiling (CAIRO2 study; validation set)

		Hepatic metastases n = 45		Extrahepatic metastases n = 35		P-value
Age	Median (range)	66.3 (36.3-77.3)		64.8 (42.4-83.6)		0.94
Gender	Male	23	51%	17	49%	0.82
	Female	22	49%	18	51%	
WHO performance status	0	29	64%	19	54%	0.37
	1	16	36%	16	46%	
LDH at randomization	Normal	21	47%	28	80%	0.002
	> ULN	24	53%	7	20%	
Site of primary tumor	Colon	11	24%	10	29%	0.89
	Rectosigmoid	18	40%	14	40%	
	Rectum	16	36%	11	31%	
Prior adjuvant therapy	No	42	93%	22	63%	0.0007
	Yes	3	7%	13	37%	
Onset metastases	Metachronous	12	27%	27	77%	<0.0001
	Synchronous	33	73%	8	23%	
Metastatic sites involved	1	45	100%	19	54%	<0.0001
	2	0		10	29%	
	>2	0		6	17%	
Diameter	Median (range)	40 (15-110)		40 (6-90)		0.86
Invasion depth	T1-2	4	9%	4	11%	0.93
	T3	34	77%	26	74%	
	T4	6	14%	5	14%	
	Unknown	1		0		
Lymph node status	N0	14	34%	10	31%	0.94
	N1	15	37%	13	41%	
	N2	12	29%	9	28%	
	Unknown	4		3		
Classification	Adenocarcinoma	41	92%	23	66%	0.003
	Mucinous carcinoma	2	4%	11	31%	
	Other	2	4%	1	3%	
Differentiation grade	Well	4	9%	6	18%	0.36
	Moderate	33	75%	20	61%	
	Poor	7	16%	7	21%	
	Unknown	1		2		
MMR status	dMMR	3	7%	5	14%	0.26
	pMMR	42	93%	30	86%	

Abbreviations: WHO = World Health Organization; LDH = lactate dehydrogenase;
 ULN = upper limit of normal; MMR = mismatch repair; dMMR = deficient mismatch repair system;
 pMMR = proficient mismatch repair system

tumor and normal DNA. The \log_2 tumor to normal signal ratio was calculated and normalized against the median value of the \log_2 ratios of all the oligonucleotides mapped to the March 2006 human reference sequence (NCBI36/hg18) on chromosome 1-22 and X. The median absolute deviation value was calculated as a quality measure of the final array CGH data. Samples from the learning and validation set with a median absolute deviation value above 0.4 were excluded.

Wave patterns occurring in the genomic profiles were smoothed with the R package NoWaves.²⁵ The R package CGHcall²⁶ was used to preprocess and normalize the data. Cellularities were set to the cellularity determined by a pathologist, and median normalization was performed. The R package DNAcopy²⁷ segmented the \log_2 ratios and they were renormalized by mode normalization. Chromosomal copy number losses and gains were identified by CGHcall, calling probabilities of 0.5 or more. The accuracy of the normalization, segmentation and calling was verified by visual inspection. To reduce the dimension of the array CGH data set without loss of information, regions were defined as previously described.²⁸

For supervised analysis, a two-sample Wilcoxon test using 10,000 permutations was performed to calculate the significance of DNA copy number differences between patients with hepatic versus extrahepatic metastases.²⁹ Two separate tests were performed to compare frequencies of gains and losses between both groups. To account for multiple testing, a permutation-based false discovery rate (FDR) was applied to the p values.

Gene dosage effects

To identify genes on the relevant chromosomal regions that show a gene dosage effect (ie, mRNA expression levels are correlated with DNA copy number status) in CRC, a data set was used from The Cancer Genome Atlas (TCGA) Data Portal (<http://tcga-data.nci.nih.gov/tcga/>) with combined mRNA and DNA copy number data. DNA copy numbers were available as segmented data, of which primary tumors with \log_2 ratios lower than -0.5 were called as 'loss' and values higher than 0.5 were called as 'gain'.

A two-sample Wilcoxon test was used to compare \log_2 mRNA expression ratios between patients with and without DNA copy number gain. To account for multiple testing, a permutation-based FDR was applied to the p values.

Immunohistochemistry on tissue microarrays (TMAs)

From the FFPE primary tumor tissues available from patients with CRC in the CAIRO and CAIRO2 study with hepatic and extrahepatic metastases, a 2 mm punch was taken to assemble TMAs. From each TMA, a 4 μ m section was mounted on glass, deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 30 min. After microwave antigen

retrieval using 10mM sodium citrate buffer (pH 6.0), slides were incubated with rabbit antibody to human polyclonal C20Orf3 (1:800, Sigma-Aldrich, St. Louis, Missouri, USA) overnight at 4°C. Subsequently, sections were incubated with Powervision Poly-HRP anti-Ms/Rb/Ra IgG (Immunologic, Duiven, The Netherlands) and developed using PowerDAB (Immunologic). The slides were counterstained with haematoxylin and evaluated by two independent observers (LJMM, MEVB). In case of discrepancy, a definite result was generated based on the expertise

Table 1. Clinicopathological features in patients with hepatic and extrahepatic metastases

		Hepatic metastases n = 182		Extrahepatic metastases n = 139		P-value
Gender	Male	129	71%	74	53%	0.002
	Female	53	29%	65	47%	
LDH at randomization	Normal	115	63%	119	86%	<0.0001
	> ULN	67	37%	20	14%	
Site of primary tumor	Colon	95	52%	56	40%	0.03
	Rectosigmoid	41	23%	49	35%	
	Rectum	46	25%	34	25%	
Prior adjuvant therapy	No	168	92%	103	74%	<0.0001
	Yes	14	8%	36	26%	
Onset metastases	Metachronous	55	30%	112	81%	<0.0001
	Synchronous	127	70%	27	19%	
Metastatic sites involved	1	182	100%	80	58%	<0.0001
	2	0		43	31%	
	>2	0		15	11%	
	Unknown	0		1		
Invasion depth	T1-2	9	5%	17	13%	0.006
	T3	138	79%	86	63%	
	T4	28	16%	33	24%	
	Unknown	7		3		
Lymph node status	N0	51	30%	55	41%	0.09
	N1	73	42%	44	33%	
	N2	48	28%	35	26%	
	Unknown	10		5		
Classification	Adenocarcinoma	151	83%	103	74%	0.02
	Mucinous carcinoma	10	5%	22	16%	
	Other	21	12%	14	10%	
Differentiation grade	Well	7	4%	5	4%	0.32
	Moderate	102	57%	68	49%	
	Poor	70	39%	66	47%	
	Unknown	3		0		

Abbreviations: LDH = lactate dehydrogenase; ULN = upper limit of normal

of a third investigator (IDN). All three investigators were blinded for the clinical and array CGH data. Stromal tissue served as a positive internal control. C20orf3 protein expression was negative if none of the tumor cells showed cytoplasmic/nuclear membrane staining, and positive if at least one tumor cell showed staining. The staining intensity was graded as negative (no staining), weak (light brown), moderate (brown) and strong (dark brown) staining. A χ^2 model was used to compare protein expression between patients with hepatic versus extrahepatic metastases. Subsequently, protein expression was also correlated with the ordinal array CGH data. All statistical tests were two-sided, and differences were considered significant when p values were below 0.05.

RESULTS

Clinical and histopathological features associated with organ-specific metastasis

Compared with patients with extrahepatic metastases (n=139), patients with hepatic metastases (n=182) were significantly more often male (p=0.002), more often had a serum LDH activity above the upper limit of normal (p<0.0001), exhibited primary tumor localization in the colon (p=0.03), showed synchronous onset of metastases (p<0.0001), and had less commonly received previous adjuvant chemotherapy (p<0.0001). There were no significant differences between patients with hepatic versus extrahepatic metastases in age (median 65 vs 63 years, p=0.39, respectively), performance status at randomisation (both groups 97% WHO 0-1) and treatment arm (both groups 47% sequential arm) (Table 1).

Primary tumors of patients with hepatic metastases more often had T3 stage (p=0.006), and mucinous histology (p=0.02) was less often observed in patients with hepatic compared with extrahepatic metastases. No significant differences between patients with hepatic versus extrahepatic metastases were observed in diameter (both median 45 mm), lymph node status, differentiation grade, and mismatch repair (MMR) status (deficient MMR in 3% vs 4%, p=0.44, respectively) (Table 1).

The prognostic value of organ-specific metastases

No significant difference in median OS was observed for patients with hepatic versus extrahepatic metastases in univariate analysis (20.3 vs 19.9 months, respectively; p=0.55). The absence of a significant impact on prognosis was confirmed for hepatic versus extrahepatic metastases in multivariate analysis (HR 0.90; 95% CI 0.66-1.23; p=0.52). The median PFS in

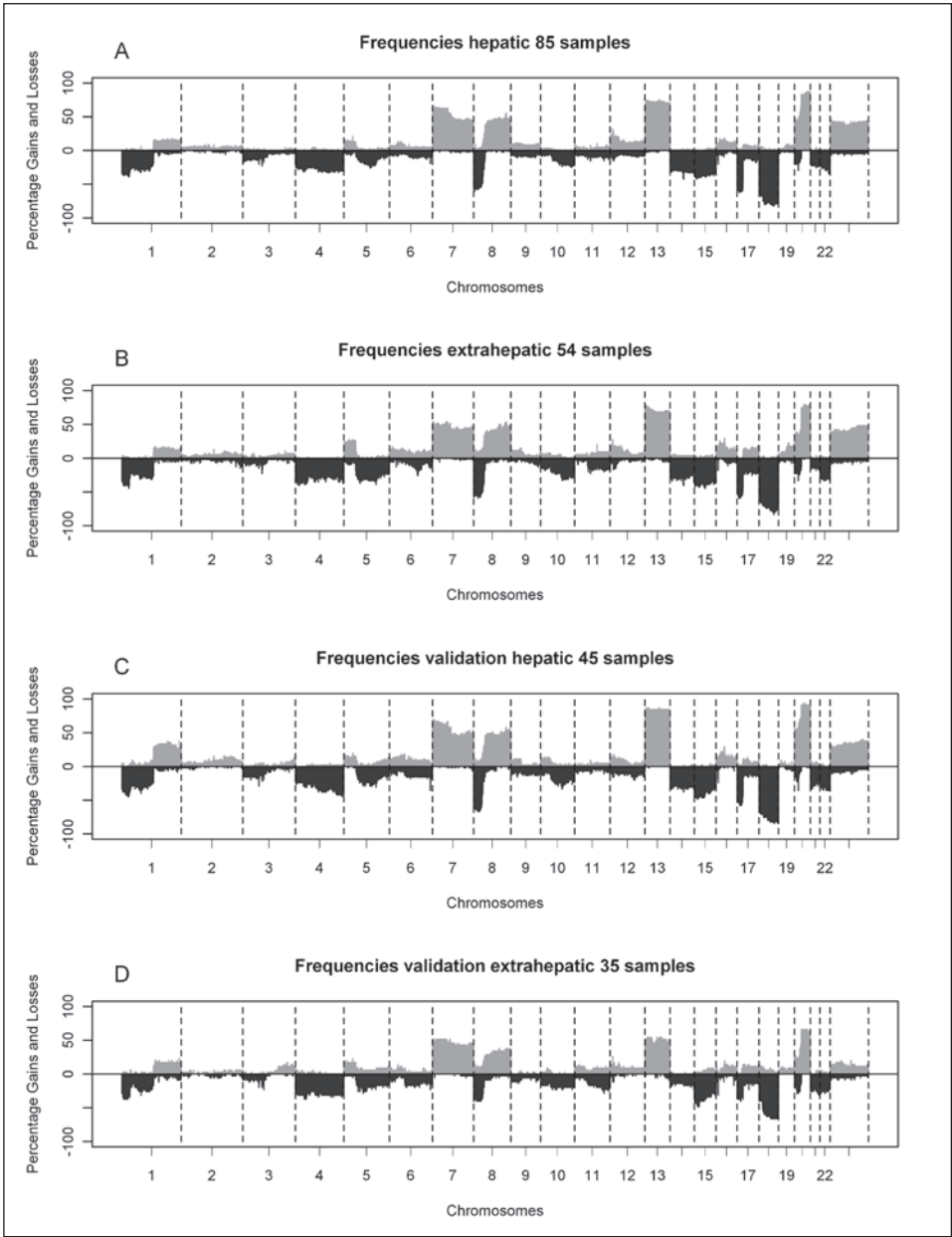


Figure 1. Genomic aberrations in CRC patients with hepatic and extrahepatic metastases. Frequency plots of DNA copy number alterations determined by aCGH in primary tumors of (A) 85 patients with hepatic versus (B) 54 patients with extrahepatic metastases (CAIRO study; learning set). Percentage gains and losses were validated in (C) 45 patients with hepatic versus (D) 35 patients with extrahepatic metastases (CAIRO2 study; validation set). The X-axis displays clones spotted on the array sorted by chromosome. The Y-axis displays the frequency of tumors with gains (above zero) or losses (below zero). Boundaries of chromosomes are indicated by dotted lines.

first-line treatment was not significantly different between patients with hepatic versus extrahepatic metastases (8.2 vs 7.0 months, respectively; $p=0.46$). A total of 291 patients were assessable for response in first-line treatment: 158 in the hepatic group and 133 in the extrahepatic group. The overall response rate (complete plus partial tumor response) in first-line treatment was significantly better in patients with hepatic metastases than patients with extrahepatic metastases (43% vs 27%, respectively; $p=0.007$). The disease control rate (complete plus partial tumor response plus stable disease) in first-line treatment was not significantly different between patients with hepatic and extrahepatic metastases (85% vs 86%, respectively; $p=0.74$).

Identification of a DNA copy number profile associated with organ-specific metastasis

The frequency plots of DNA copy number aberrations throughout the genome in patients with hepatic ($n=85$) and extrahepatic ($n=54$) metastases were very similar (Figure 1). Despite the high concordance level between the two groups, small differences in DNA copy number profile were observed. Patients with hepatic metastases had significantly more gains at 20p11 than patients with extrahepatic metastases ($p<0.05$; FDR = 0.88)(Supplemental Table 3).

Supplemental Table 3. Percentage copy number gains of chromosomal regions of 85 patients with hepatic versus 54 patients with extrahepatic metastases (CAIRO study; learning set)

Chromosome	Bp start	Bp end	Hepatic n=85	Extrahepatic n=54	P-value	FDR
7p15.2-p15.1	27225	28185	62.4%	42.6%	0.03	0.88
17q22-q23.2	53388	55856	4.7%	16.7%	0.04	0.88
19q13.13-q13.2	43029	45196	8.2%	22.2%	0.04	0.88
19q13.2	45309	46141	5.9%	20.4%	0.02	0.88
20q13.2-p11.22	20983	21652	54.1%	33.3%	0.02	0.88
20p11.21	22354	22994	57.6%	38.9%	0.04	0.88
20p11.21	23010	23234	58.8%	38.9%	0.03	0.88
20p11.21	23248	23821	60.0%	38.9%	0.02	0.88
20p11.21	23841	23925	60.0%	40.7%	0.03	0.88
20p11.21	23945	24324	61.2%	42.6%	0.04	0.88
20p11.21	24345	24637	64.7%	40.7%	0.006	0.88
20p11.21	24651	25235	64.7%	44.4%	0.02	0.88

Abbreviations: Bp = base pair; FDR = false discovery rate

Supplemental Table 4. Percentage copy number losses of chromosomal regions of 85 patients with hepatic versus 54 patient extrahepatic metastases (CAIRO study; learning set)

Chromosome	Bp start	Bp end	Hepatic n=85	Extrahepatic n=54	P-value	FDR
2p21-q35	45612	217250	0%	7.4%	0.04	0.55
2q35	217262	217702	1.2%	11.1%	0.03	0.51
2q35	217729	218518	1.2%	13.0%	0.01	0.51
2q37.1	232927	232995	3.5%	14.8%	0.04	0.54
5q11.2	50533	51478	7.1%	24.1%	0.01	0.51
5q11.2	51498	52584	8.2%	24.1%	0.02	0.51
5q11.2	52607	54776	8.2%	25.9%	0.01	0.51
5q11.2	54800	54868	9.4%	24.1%	0.04	0.54
5q11.2	54887	56243	9.4%	25.9%	0.02	0.51
5q35-q12.1	56254	58905	10.6%	29.6%	0.01	0.51
5q12.1	58921	59531	12.9%	31.5%	0.02	0.51
5q12.1	59548	59781	11.8%	31.5%	0.009	0.51
5q12.1	59799	59884	12.9%	31.5%	0.02	0.51
5q12.1	59911	61912	10.6%	29.6%	0.01	0.51
5q12.1	61936	62402	11.8%	27.8%	0.03	0.51
5q12.3	64013	65193	14.1%	31.5%	0.03	0.51
5q12.3	65218	66277	15.3%	33.3%	0.02	0.51
5q35-q13.1	66292	67610	16.5%	35.2%	0.02	0.51
5q13.1	67614	67789	16.5%	37.0%	0.01	0.51
5q37.1-q13.2	67811	69274	16.5%	35.2%	0.02	0.51
5q13.2	70623	70763	14.1%	29.6%	0.04	0.55
5q11.2-q14.1	70785	79782	16.5%	33.3%	0.04	0.54
5q31.1	131772	131866	20.0%	38.9%	0.03	0.51
5q31.1	131893	133685	16.5%	33.3%	0.04	0.54
5q31.2	138632	138781	12.9%	29.6%	0.03	0.51
5q31.3	139498	139660	11.8%	27.8%	0.03	0.51
5q31.3	139674	140933	11.8%	29.6%	0.02	0.51
5q31.3	140947	143033	11.8%	27.8%	0.03	0.51
5q11.2-q32	143045	146785	10.6%	25.9%	0.03	0.51
5q11.2-q33.1	146933	148722	9.4%	25.9%	0.02	0.51
5q33.1	148726	148735	9.4%	29.6%	0.007	0.51
5q33.1	148749	149538	10.6%	27.8%	0.02	0.51
5q11.2-q33.2	149551	153853	9.4%	25.9%	0.02	0.51
5q11.2-q35.1	154550	172078	8.2%	22.2%	0.04	0.54
5q11.2-q35.3	172103	180154	7.1%	22.2%	0.02	0.51
5q35.3	180168	180356	9.4%	24.1%	0.04	0.54
5q35.3	180365	180533	5.9%	22.2%	0.01	0.51
5q35.3	180548	180645	7.1%	24.1%	0.01	0.51
6q12.1-q22.1	112779	114303	9.4%	24.1%	0.03	0.51
9q12.1-q12	38471	69214	9.4%	0%	0.03	0.51
10q11.21	42937	44189	5.9%	18.5%	0.04	0.54
10q12.1-q11.22	44197	46568	7.1%	20.4%	0.04	0.54
16p13.3	5675	6013	10.6%	0%	0.02	0.51
17q21.2	35826	36110	9.4%	24.1%	0.04	0.54

Abbreviations: Bp = base pair; FDR = false discovery rate

Loss at 5q12 was significantly less commonly observed in patients with hepatic versus extra-hepatic metastases ($p < 0.05$; FDR = 0.54) (Supplemental Table 4).

To validate these differences in DNA copy number profiles, an additional independent validation set of 45 patients with hepatic metastases and 35 patients with extrahepatic metastases was selected. Significantly more gains at 20p11 in patients with hepatic versus extrahepatic metastases were confirmed (FDR < 0.05) (Figure 1; Supplemental Table 5). Differences in copy number aberrations at 5q12 could not be validated.

Identification of differentially expressed genes at 20p11

To determine the most relevant genes at 20p11 with a potential role in hepatic-specific CRC metastasis, we used a publicly available dataset of 141 CRC patients. For these patients, both DNA copy number and gene expression profiling of the primary tumor were available. Putative genes with a dosage effect were identified by comparing tumors with 20p11 gain to tumors without 20p11 gain. This approach revealed 12 out of 28 genes with expression levels that were significantly influenced by the occurrence of 20p11 gain, namely *XRN2*, *NXT1*, *GZF1*, *NAPB*, *CSTL1*, *CST3*, *CST5*, *C20orf3*, *ACSS1*, *ENTPD6*, *PYGB*, *ABHD12* (Supplemental Table 6). Of these 12 differentially expressed genes, *C20orf3* showed the strongest correlation between copy number status and RNA expression (FDR < 0.0001) (Figure 2).

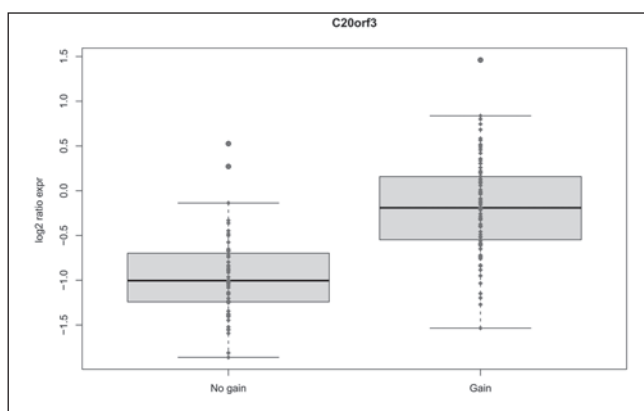


Figure 2. *C20orf3* mRNA expression correlated with DNA copy number. Box plot of *C20orf3* mRNA expression in 141 patients with and without gain of 20p11. A strong correlation was observed between copy number status and RNA expression (FDR < 0.0001).

Supplemental Table 5. Percentage copy number gains of chromosomal regions of 45 patients with hepatic versus 35 patients with extrahepatic metastases (CAIRO2 study; validation set)

Chromosome	Bp start	Bp end	Hepatic n=45	Extrahepatic n=35	P-value	FDR
20p11.23-p11.22	20983	21652	68.9%	34.3%	0.005	0.01
20p11.21	22354	22994	71.1%	34.3%	0.002	0.01
20p11.21	23010	23234	68.9%	40.0%	0.02	0.02
20p11.21	23248	23821	68.9%	40.0%	0.02	0.02
20p11.21	23841	23925	68.9%	40.0%	0.02	0.02
20p11.21	23945	24324	68.9%	40.0%	0.02	0.02
20p11.21	24345	24637	71.1%	40.0%	0.01	0.02
20p11.21	24651	25235	71.1%	42.9%	0.02	0.02

Abbreviations: Bp = base pair; FDR = false discovery rate

Supplemental Table 6. Differences in mRNA expression of genes located at 20p11 in patients with and without gain of 20p11

Chromosome	Bp Start	Bp End	Gene	P-value	FDR
20p11.23-p11.22	20983	21652	XRN2	<0.0001	<0.0001
20p11.21	22354	22994	NXT1	<0.0001	<0.0001
20p11.21	23010	23234	GZF1	<0.0001	<0.0001
20p11.21	23248	23821	NAPB	<0.0001	<0.0001
20p11.21	23841	23925	CSTL1	<0.0001	<0.0001
20p11.21	23945	24324	CST3	<0.0001	<0.0001
20p11.21	24345	24637	CST5	<0.0001	<0.0001
20p11.21	24651	25235	C20orf3	<0.0001	<0.0001
20p11.23-p11.22	20983	21652	ACSS1	<0.0001	<0.0001
20p11.21	22354	22994	ENTPD6	<0.0001	<0.0001
20p11.21	23010	23234	PYGB	0.0007	0.002
20p11.21	23248	23821	ABHD12	<0.0001	<0.0001

Abbreviations: Bp = base pair; FDR = false discovery rate

Confirmation of differential expression by immunohistochemistry

Using the TMAs of both CAIRO and CAIRO2 patients, C20orf3 expression could be determined in 581 patients, of which 325 had hepatic metastases and 283 extrahepatic metastases. Examples of C20orf3 expression in TMA cores of primary adenocarcinomas were shown in Figure 3. In situ confirmation of C20orf3 protein expression yielded a higher percentage of primary tumors with presence of C20orf3 staining in patients with hepatic versus extrahepatic metastases (59% versus 41%, respectively; $p=0.01$)(Table 2). In 199 out of the 219 patients with copy number data, copy number status was correlated with C20orf3 expression. A significant positive correlation of C20orf3 protein expression with the ordinal array CGH ratios was showed as well ($p<0.0001$)(Table 3). Validation of other genes was hampered by the unavailability of adequate antibodies for immunohistochemistry on FFPE tissues.

Table 2. C20orf3 protein expression in correlation with organ-specific metastasis

	C20orf3 protein expression			P-value
	Absence	Presence	Total	
Hepatic metastases	50 (45%)	275 (59%)	325	0.01
Extrahepatic metastases	61 (55%)	195 (41%)	256	
Total	111	470	581	

C20orf3 protein expression in primary colorectal tumors in patients with hepatic and extrahepatic metastases by immunohistochemistry on TMAs

Table 3. C20orf3 protein expression in correlation with genomic aberrations at 20p11

	C20orf3 protein expression			P-value
	Absence	Presence	Total	
Loss	21 (50%)	2 (1%)	23	<0.0001
No CNA	18 (43%)	45 (29%)	53	
Gain	2 (5%)	102 (65%)	104	
Amplification	1 (2%)	8 (5%)	9	
Total	42	157	199	

C20orf3 protein expression in primary colorectal tumors correlated with the genomic aberrations at chromosome 20p11. In 20 patients with a known copy number status, C20orf3 expression was not accessible due to an insufficient amount of tumor cells on the TMA. Abbreviation: CNA = copy number aberration

DISCUSSION

In this study we characterized specific clinicopathological features and genomic aberrations of the primary tumor in patients with CRC with hepatic versus extrahepatic metastases.

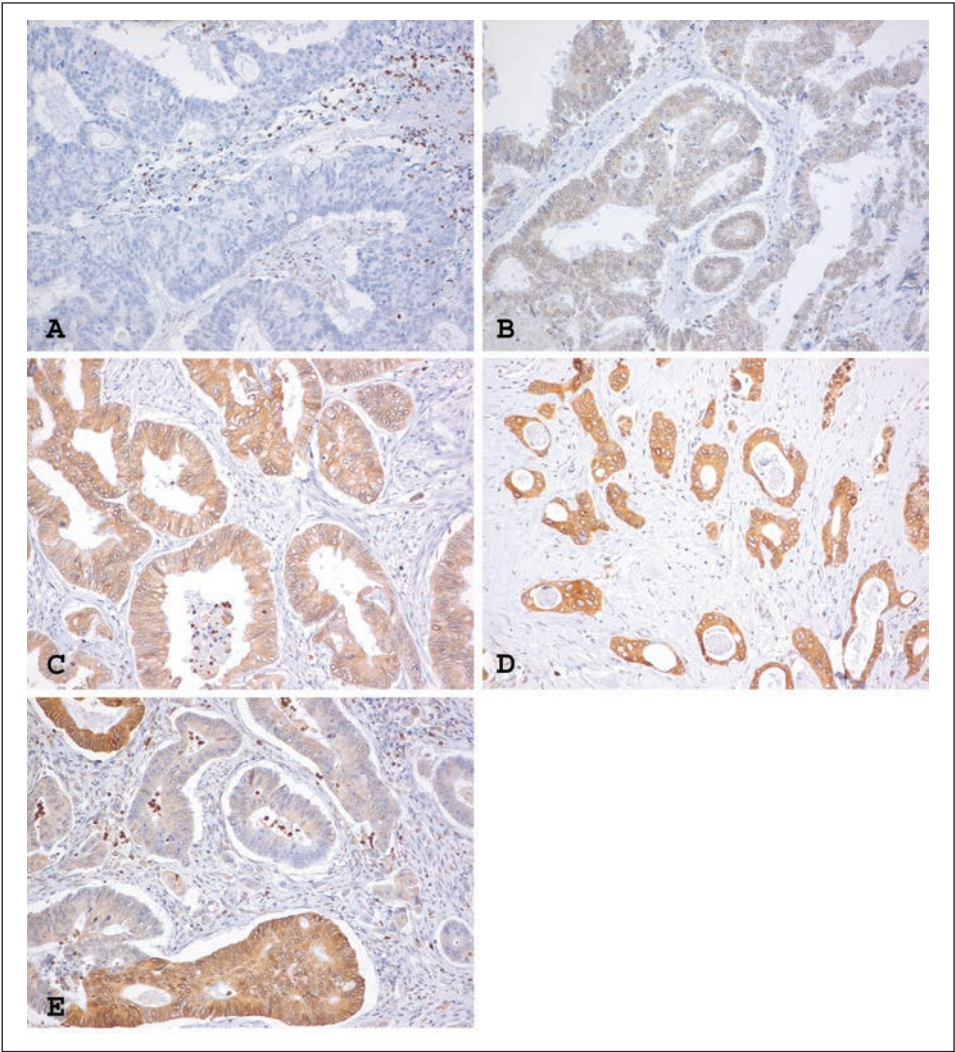


Figure 3. C20orf3 protein expression. Examples of C20orf3 protein expression in TMA cores of primary colorectal adenocarcinomas showing no expression (A), weak expression (B), moderate expression (C), strong expression (D) and heterogeneity of expression (E).

In patients with hepatic metastases, the primary tumor was more often in the colon. The venous drainage of the colon is via the portal system, therefore the liver has always been regarded as the first site of hematogenous spread. The increased incidence of extrahepatic metastases in rectosigmoid and rectal carcinoma can be attributed to the direct hematogenous spread into the systemic circulation via the inferior and middle rectal veins. However, over one-third of patients with colon carcinomas develop extrahepatic metastases. This supports a substantial role for features that are inherent to the cancer cell and the micro-environment. In the present study, liver-specific metastasis was more often observed in male than female patients. This observation was also reported in a smaller cohort of patients³⁰, but a clear explanation is lacking. The onset of metastases is another clinical feature associated with site-specific metastasis. The majority of patients with stage IV disease present with hepatic metastases at diagnosis, and only one-sixth of the patients had extrahepatic metastases. These differences may partly be related to the diagnostic procedures, since extrahepatic metastases may be more difficult to detect. Indeed, progress has been made in diagnostic techniques, which may explain the rising incidence of synchronous pulmonary metastases from CRC.³¹ The third clinical feature associated with site-specific metastasis is serum LDH. Our results confirm that this variable is not only a surrogate estimate for tumor burden, but also a serological factor for hepatic metastases.³²

Pathological examination of CRC resection specimens identified T stage and tumor type, which are correlated with organ-specific metastases. T1 and T2 tumors do not often metastasize, but, if distant spread occurs, they are more likely to arrest outside the liver. These tumors are located in the mucosa or submucosa, which have the greatest density of lymphatic vessels.³³ We hypothesize that T1 and T2 tumors metastasize via these lymphatic vessels, thereby escaping entrapment in the liver and producing outgrowth in extrahepatic organs. Mucinous adenocarcinomas are a less common histological subset of CRC, also appearing to metastasize more often in extrahepatic organs. These tumors probably possess specific traits that stimulate invasion in extrahepatic organs, but the underlying mechanism is unknown.

Several prognostic factors have been investigated in metastatic CRC, but the influence of the metastatic site as an additional predictor of outcome is highly controversial. Despite differences in clinicopathological features between patients with hepatic and extrahepatic metastases, the median OS and PFS were not significantly different between the groups. Even after correction for multiple prognostic factors, established in the same study population in a previous study³⁴, metastatic site is not an independent prognostic factor for survival in CRC. However, we observed a higher overall response rate to first-line chemotherapy in patients with hepatic metastases than in those with extrahepatic metastases. First, there may be a bias in response assessments, since liver metastasis may be easier to assess than extrahepatic metastases. Second, significantly more patients with extrahepatic metastases were treated with previous adjuvant chemotherapy, while patients with hepatic metastases were not. This could

in theory have resulted in a (partial) resistance to chemotherapy in the former group. However, the differences in overall response rates between patients with hepatic and extrahepatic metastases did not translate into differences in survival. This is in line with a previous study by our group³⁴ in which we established that the application of previous adjuvant chemotherapy was not of prognostic value in the same patient cohort.

Genomic aberrations responsible for CRC metastasis to the liver remain speculative and poorly understood. Most of the existing data were obtained using experimentally derived mouse tumor models. In the present study, we aimed to investigate genomic aberrations that drive CRC cells to the liver directly from human samples. The comparison of primary tumors derived from patients with hepatic and extrahepatic metastases identified more gains at 20p11 in patients with hepatic metastases. In contrast to 20q, the correlation of 20p11 with liver metastasis has not previously been described. Gain of 20q is a common genomic aberration in CRC and an indicator of poor prognosis³⁵ and metastatic potential.³⁶ Chromosome 20q gains are more often observed in primary tumors that metastasize to the liver compared to tumors that metastasize to the peritoneum and tumors without distant metastases.³⁰ The design of our study and our patient selection are different, as we exclusively used tumors that possess metastatic potential. Therefore, differences in gain of 20p11 are more likely to be attributable to organ-specific metastasis. Next, our array contained 180880 nucleotides, and an adequate number of samples was analyzed, therefore we expect an increased power in our analysis. However, our results only suggest a correlation between 20p11 gain in the primary tumor and liver metastases. We should keep in mind that primary tumors are highly heterogeneous in terms of both their cell populations and their ability to metastasize. Therefore, it may be that the genes responsible for organ tropism might not be detected in the bulk of the primary tumor. A next step could be to search directly for genes involved in organ-specific metastases by profiling metastatic samples from different secondary sites in relation to their primary tumor.

The number of genes at 20p11 is too high to really disclose those that play a role in organ-specific metastases. Not all genes mapping at gained regions are recurrently overexpressed; therefore we compared gene expression between tumors with and without 20p11 gain. Of the 12 out of 28 genes at 20p11 with a dosage effect on expression, *C20orf3* showed the strongest correlation, and protein expression was associated with hepatic-specific metastases. *C20orf3* is a member of the lactonohydrolase super family, and the potential involvement of this protein in enzymatic processes is suggested.³⁷ Protein expression has mainly been demonstrated in the liver, but no relation to hepatic-specific metastases has yet been reported. In vitro assays should provide evidence for a causal role of this gene in metastasis formation. In addition, confirmation of their relevance to liver-specific metastasis can be made by showing functional evidence of the pro-liver-metastatic effect in a xenograft model. It is interesting that *C20orf3* maps on the opposite allele at a distance of a few kilobases from the human *CMAF* gene, which is correlated with liver metastases.³⁸ However, in the publicly available dataset we used,

CMAF was not overexpressed in 20p11 gained primary tumors. Expression of *XRN2*, *NXT1*, *GZF1*, *NAPB*, *CSTL1*, *CST3*, *CST5*, *ACSS1*, *ENTPD6*, *PYG* and *ABHD12* was also increased in primary tumors with 20p11 gain, but we could not analyze the correlation of their protein expression with organ-specific metastases. However, if 20p11 gain influences organ-specific metastases, this could well be caused by altered expression of multiple genes rather than a single gene.

Organ-specific metastasis is a highly complex process, and the capacity to disseminate also depends on additional features other than chromosomal aberrations at chromosome 20p11. First, smaller alterations including somatic mutations are probably important, and novel technologies such as next-generation sequencing will play a pivotal role. Germline variants detectable by genome-wide association studies might also contribute to organ-specific metastasis, but have not yet been identified. Second, the barriers to infiltrating an organ depend on the architecture and specific features of the microenvironment. Endothelial adhesive interactions and certain aspects of the vasculature have been proposed to contribute to dissemination in specific organs.³⁹ Some studies suggest that the secretion of cytokines results in a pro-metastatic microenvironment,^{40,41} but these data need further characterization in cancer models.

In conclusion, an array CGH profile including the protein-encoding gene *C20orf3* was over-represented in primary CRCs that preferentially metastasize to the liver. Although selected from a large clinical trial, it should be realized that our results are derived from a retrospective analysis. Therefore selection bias cannot be excluded, and prospective studies on this topic are warranted. Furthermore, the possible role of this liver metastasis-associated gene in specific steps of the hepatic metastatic process needs to be functionally validated. This could result in the development of (1) new prognostic markers that could help in identifying patients who are most likely to develop liver metastases and (2) liver-specific anti-metastatic therapies for the future.

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Chapter 10

General discussion and summary

GENERAL DISCUSSION

Only a limited number of patients with metastatic CRC are candidates for curative resection, the majority of patients present with unresectable disease. Survival benefit in these patients can be achieved with systemic treatment including cytotoxic and targeted agents. These targeted agents, which in the treatment of CRC concern anti-VEGF and anti-EGFR antibodies, have further increased the life expectancy of metastatic CRC patients. However, despite this increase in median survival, the heterogeneity in survival rates between patients is striking. In this thesis we attribute this heterogeneity in survival to a number of prognostic and predictive factors, which should tailor patient care and improve survival in individual patients. In addition, to encourage the development of new targeted therapies and subsequent improvement of survival, we aimed to further define the (epi)genetic alterations which are involved in the metastatic cascade.

Clinical applicable nomogram for predicting prognosis

Metastatic CRC represents a heterogeneous group of tumors. Until now an extraordinary amount of research has focused on differentiating the good, the bad and the ugly tumors. Most of these studies concerned retrospective analyses in small patient cohorts that were not externally validated. Currently, clinicians have a hard time in finding their way among all the prognostic markers that have been presented in CRC patients. However, it is critical that healthcare professionals are familiar with the impact of clinicopathological features on the outcome of their individual patients. Obviously, no single factor can account for the wide variability in outcomes, as is observed among individual patients. A variety of clinical and pathological features have been identified as prognostic markers in retrospective analysis of clinical trials. In our clinical studies (CAIRO and CAIRO2) we have confirmed most of these well-known prognostic factors, like serum LDH, number of lymph nodes retrieved, and number of metastatic sites involved. However, we also investigated several other clinical and pathological features of which the prognostic value is less well established. We demonstrated that the onset of metastasis (synchronous versus metachronous) does not influence clinical outcome, but that the histology of the primary tumor is of substantial importance in predicting the prognosis of metastatic CRC patients.

The research on prognostic markers in this thesis should initiate additional studies that aim to determine the most strongest prognostic factors and incorporate them in a model that can be used in clinical practice. Nomograms (prognostic models) that combine patient survival factors are useful clinical tools when determining prognosis and subsequent tailoring of the most appropriate treatment options. These algorithms could also be used by investigators to

guide the inclusion of patients in clinical trials, and prevent heterogeneity in outcome. In other tumor types, like renal cell carcinoma, nomograms have been identified as user-friendly and provide the clinician and the patient a tool to predict a specific outcome over time.

A nomogram predicting the prognosis for CRC patients with irresectable metastases should be generated by combining the outcome data of multiple randomized clinical trials. These clinical trials should be carefully selected because prognosis can be influenced by differences in best supportive care among various countries as well as over time, and also by differences in reimbursement systems. Before generating a nomogram it is essential to establish how extensive this algorithm should be. It should be realized that a nomogram must be user-friendly and that markers need to be easily determined in general practice. After establishing these conditions, the relevant prognostic markers should be prospectively evaluated in a large test cohort with proper validation in an independent dataset.

Predictive value and targeting of mutant *KRAS* in more detail

Nowadays, anti-EGFR therapy is restricted to patients with a *KRAS* wild-type tumor. The *KRAS* mutation as a negative predictive marker for response to anti-EGFR therapy is one of the most important discoveries in CRC in recent years. An activating point mutation in *KRAS* results in a 12-fold increase of the RAS-GTPase activity, independent of binding of the epidermal growth factor to the EGFR. Obviously, a point mutation is not the only mechanism that can alter *KRAS* protein expression. Epigenetic factors, genomic aberrations, single nucleotide polymorphisms, and dysregulation of transcription factors may also regulate *KRAS* activity. In this thesis we correlate *KRAS* copy number aberrations and miRNAs that target *KRAS* with clinical outcome to cetuximab in metastatic CRC patients. These results suggest that the clinical effects of *KRAS* are the result of a complex interaction of several regulatory mechanisms beyond the *KRAS* point mutation status. However, our study is only hypothesis-generating and additional analyses should be performed. Firstly, our data require an independent validation, which should also include patients who do not receive anti-EGFR therapy. Secondly, to get insight in the effects of miRNAs and copy number aberrations at the *KRAS* protein level, functional studies should be performed measuring RAS-GTPase activity and *KRAS* protein expression.

The correlation between miRNAs that inhibit the translation of *KRAS* and the response to cetuximab is of special interest because it can result in the development of new treatment strategies. Targeting *KRAS* is probably only relevant in patients harboring an activating point mutation in *KRAS* because of the elevated levels of RAS-GTPase activity. Mutations in *KRAS* have been found in 30-50% of metastatic CRC patients. Therefore, silencing of mutant *KRAS* by miRNAs has become an attractive therapeutic strategy in metastatic CRC patients. Recently, an *in vitro* study showed that miRNAs targeting *KRAS* efficiently reduced *KRAS* expression. Subsequent silencing of *KRAS* by miRNAs *in vivo* showed pronounced antitumor effect without

substantial side effects.¹ These promising animal studies should stimulate researchers to investigate which miRNA is the most potential silencer of *KRAS* in human samples, which should be further analyzed as a new treatment modality in *KRAS* mutated metastatic CRC patients.

***KRAS* oncogene as a model to study metastasis**

Because of the increased use of anti-EGFR therapy in metastatic CRC patients, a test for *KRAS* mutation status is frequently determined in tumor tissue. *KRAS* mutation analysis is usually performed on primary tumor tissue since metastatic tissue is often not available. Previous studies were inconclusive whether the result of the primary tumor corresponds with that of metastatic tissue, and some investigators suggested to routinely perform a mutation analysis in both primary tumor and metastatic tissue. In this thesis we present the largest study on this topic to date, involving 305 CRC patients, and we demonstrate that the *KRAS* mutation status is highly concordant between primary tumors and liver metastases. The implication of these results for general practice is that tissue of both the primary tumor and liver metastases may be used for *KRAS* mutation testing.

Since *KRAS* mutation is an early event during colorectal carcinogenesis, an identical *KRAS* mutation status between primary tumors and distant metastases is to be expected as in our study on primary tumors and matched liver metastasis. Surprisingly, others have shown (although in small series) a substantial discordance in *KRAS* mutation status between the primary tumor and regional metastatic lymph nodes, which questions the role of lymphatic spread in relation to hematogenous dissemination. Traditionally, regional lymph nodes are considered to have an important role in the metastatic process, and both staging and the application of adjuvant chemotherapy depends on lymph node involvement. It is yet unexplained how lymph node metastases can lose or acquire a mutation in *KRAS*. One hypothesis is that alternative pathways of metastases, such as vascular invasion and perineural growth, might be more important in CRC liver metastases. Therefore, additional analyses should be performed using the human *KRAS* oncogene as a model to study metastases. Firstly, *KRAS* mutation status should be determined and compared in areas of perineural growth and vascular invasion within the primary tumor. Secondly, these areas of vascular invasion, perineural growth, and regional lymph nodes of the primary tumor should also be investigated in patients with other metastatic organs involved, like the lungs and the peritoneal cavity. Identification of CRC dissemination pathways is clinically important for an adequate tailoring of adjuvant systemic treatment in stage II and III CRC patients.

From dissemination to colonization: the role of (epi)genetics

The transition from colorectal hyperplasia to adenoma and subsequent invasive carcinoma is characterized by the acquisition of specific genetic alterations (*APC*, *KRAS*, *TP53*, *TGFB*). This model of stochastic mutational events has found widespread acceptance. Once the colorectal tumor becomes invasive, metastatic progression can proceed rapidly without latency resulting in synchronous metastases. The relatively short latency of most of the metastatic relapses in CRC implies that the metastatic competence is already acquired during the early stage of malignant transformation. Indeed, our data showed that overt metastases and primary tumors share similar genomic aberration patterns, implying that most of the metastatic traits are already present in the primary tumor. Genetic evolution within the primary tumor is probably necessary to generate cell populations with increased fitness, because even cancer cells that are fully neoplastic have extremely low chances of proliferating at sites of dissemination. This makes it very unlikely that precursor cells of overt metastases disseminate early to sites where they proceed to undergo their own divergent genetic evolution (parallel progression model). However, oncogenic transformation is probably not completely sufficient for metastatic competence, and tumor cells must acquire some additional abilities to surmount the natural barriers against metastases. Indeed, our metastases showed few non-recurrent additional genetic aberrations which were absent in the corresponding primary tumor. Due to the non-recurrence of the observed additional genomic aberrations, this (last) part of the metastatic outgrowth cannot be generalized to all CRC patients.

In some CRC patients metachronous metastases occur after a prolonged latency period. The presence of disseminated tumor cells (DTCs) in patients whose primary tumors have been removed correlated with metastatic relapse. Malignant cells that disseminate can reside as single cells or micrometastatic clusters, mainly in the blood, lymph nodes or bone marrow. The latent DTCs either lack the ability to colonize or are prevented to colonize by the microenvironment. (Epi)genetic dysregulation of a latent DTC population probably combined with local changes in the microenvironment endow surviving DTCs towards full metastatic competence at secondary sites. However, it remains unclear when these DTCs abandon the primary tumor and if they need additional traits for outgrowing at secondary sites. Progress in understanding the origin and fate of DTCs, by characterizing the (epi)genetic aberration in DTCs in respect to the primary tumor and the metastases, will offer new insight into the process of metastases. Within clinical oncology new targeted treatments are being developed for metastatic disease, and for several reasons it is important that we established that genetic aberrations between primary CRC and metastases were highly similar. Firstly, comparing genetic profiles contribute to the determination which tumor tissue will best predict treatment outcome. Current clinical practice can proceed using archived material of the primary tumor to determine the constitution of the molecular target to select patients for treatment of the metastasis. Secondly, instead of focusing on additional genetic features responsible for metastatic formation, identification

and understanding of the metastases-associated genes in the primary tumor is probably more important and should be the route to the discovery of new drug targets.

Intrinsic tumor cell properties and organ-specific metastases

Colorectal tumors predominantly spread to the liver in 80% of patients with recurrent disease. The mesenteric circulation from the bowel and the permissiveness of the liver capillary sinusoids are thought to favor liver metastases. Following circulatory patterns from the liver or directly from primary tumors in the descending colon and rectum, the second most common site of metastases is the lungs. However, in addition to the influence of hematogenous dynamics, colon carcinoma cells preferentially adhere to the liver and lung endothelia, suggesting the existence of specific molecular interactions that favor the retention of tumor cells in these organs. This ‘homing’ process is probably the result of both intrinsic tumor cell properties and organ microenvironment, and might be provided by (epi)genetic dysregulation that provide a selective growth advantage. The dysregulation of genes in tumor cells could already be prominently expressed in a primary tumors, although their unique role becomes obvious at a specific distant site.

The role of chromosome 20p11 in liver-specific metastases is suggested in this thesis which emphasizes the ‘seed and soil’ theory of Paget. The function of the most prominently overexpressed gene (*C20orf3*) in patients with 20p11 gain is not clear. Functional *in vitro* assays should provide evidence for a causal role of this gene in metastasis formation. In addition, confirmation of their relevance to liver-specific metastases can be made by showing functional evidence of the pro-liver-metastatic effect in a mouse model. Translation of these findings to the clinic should stimulate the development of targeted therapeutics to prevent such organ-specific metastases.

Next to the clinical implications of chromosome 20p11 in organ-specific metastases, this aberrant region could also be used to gain insight in the dissemination pathways of CRC. In situ hybridization of chromosome 20p11 in the primary tumor with regions of vascular invasion, perineural growth, and lymph node involvement visualizes the pathway of tumor cells with and without 20p11 aberrations. We could also demonstrate tumor cell heterogeneity, by comparing in situ hybridization analysis of chromosome 20p11 in the metastasis and the primary tumor. It is well-known that tumors are heterogeneous and contain numerous subpopulation of cells that have a different metastatic potential. However, heterogeneity in ‘homing’ associated chromosomal aberrations has not yet been investigated.

In the past decennia, management of metastatic CRC has mainly focused on patient and histological characteristics, which were completed with data from the laboratory and radiological imaging. These traditional parameters are still relevant, but recent advances in human genetics

and proteomics enabled us to a more detailed understanding of metastatic CRC. The translational studies performed in this thesis offer new insights into the intriguing process of CRC metastases, and are a step forward into the era of personalized medicine.

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SUMMARY

Most colorectal tumors develop as benign lesions and progress to more invasive phenotypes when the appropriate (epi)genetic alteration in oncogenes and tumor suppressor genes occur. This process also involves the selection of traits that are advantageous to disseminating cancer cells allowing them to colonize a secondary site, resulting in metastases. Yet, it is still a matter of debate how tumor cell populations evolve to acquire molecular alterations in metastases-associated genes. Unraveling the question how, when, and where the precursors of overt metastases arise, will give insight in the clinical opportunities to target and prevent metastatic disease. However, currently there are no curative options in case of permanently irresectable metastatic disease, but a significant and clinically relevant benefit in survival can be achieved with systemic treatment. Heterogeneity in response rates and overall survival are mainly due to differences in the presence of prognostic and predictive markers. More clarity about prognostic and predictive markers could individualize follow-up and treatment of metastatic CRC patients.

In **chapter 2** we describe the prognostic value of the number of examined lymph nodes in 1227 rectal cancer patients who were selected from a multicenter prospective randomized trial investigating the value of short-term neoadjuvant radiotherapy. In these patients we also determined which factors relate to the number of lymph nodes found and the presence of lymph node metastases. The median number of examined lymph nodes in all patients was 7. The number of retrieved lymph nodes was significantly higher in patients with node-positive compared to node-negative patients. After short-term neoadjuvant radiotherapy fewer lymph nodes were retrieved compared to the surgery only group. Substantial variance was found between the 49 participating laboratories, with median yields varying between 1.5 and 13.5 lymph nodes. These variations in lymph node yield between pathology laboratories and individual pathologists were striking. The following patient and tumor characteristics were associated with a significant lower lymph node retrieval: age over 60 years, obesity, small tumor size, low invasion depth, poor differentiation grade, and absence of a lymphoid reaction surrounding the tumor. Node-negative patients in whom 7 or less lymph nodes were examined had a smaller recurrence free interval compared with patients in whom at least 8 lymph nodes were examined. We concluded that the number of retrieved lymph nodes after total mesorectal excision surgery is partially dependent on biological features, and is also influenced by the choice of treatment and pathological assessment of the specimen. Our data indicate that a median number of 8 or more examined lymph nodes is reliable for adequate staging in patients with rectal cancer.

Mucinous adenocarcinomas are a rare histological subtype of CRC (5-15%), in which the tumor cells secrete abundant extracellular mucin involving more than 50% of the tumor volume. The comparison of clinicopathological features and outcome in 1010 metastatic CRC patients with mucinous adenocarcinomas and the more common adenocarcinomas are presented in **chapter 3**. Patients with mucinous adenocarcinomas (n=99) were older, more often had a normal serum LDH, extrahepatic localization of metastases, a larger primary tumor diameter, and a higher T stage compared to patients with adenocarcinomas (n=911). A deficient mismatch repair system and *BRAF* mutations were more frequently observed in patients with mucinous adenocarcinomas versus adenocarcinomas. OS, PFS, and overall response rates were significantly worse for patients with mucinous adenocarcinomas compared to adenocarcinomas. In multivariate analysis, mucinous histology was a negative prognostic factor for OS resulting in a combined hazard ratio of 1.78 (95%CI 1.35-2.35). This strong negative prognostic value of mucinous adenocarcinomas supports the use of this histopathological feature as a stratification factor for randomized clinical trials in metastatic CRC.

In **chapter 4** we report the differences in clinicopathological features and outcome in 550 CRC patients with metachronous (beyond 6 months of the primary diagnosis) versus synchronous (within 6 months of the primary diagnosis) metastases. The clinical and pathological characteristics associated with poor prognosis that we observed more often in patients with synchronous metastases (n=280) versus metachronous metastases (n=270) concerned an abnormal serum LDH concentration, a worse WHO performance status, primary tumor localization in the colon, and a higher T stage. Unexpectedly, we observed no significant difference in median OS and PFS between patients with synchronous and metachronous metastases. A possible explanation includes a (partial) chemo resistance in patients with metachronous disease because of previous adjuvant systemic treatment. Possible differences in screening procedures resulting in a lead time bias to diagnosis or in prognostic molecular markers between patients with synchronous versus metachronous metastases as a cause for this finding remains speculative. Comparing our results with the literature, most studies observed a worse OS for patients with synchronous versus metachronous metastases. However, our analysis differs from the published literature in one important aspect, in that only patients with a prior resection of the primary tumor were included in the synchronous group. If patients with both resected and non-resected primary tumors were included in the synchronous group, a significant worse median OS was observed for patients with synchronous versus metachronous metastases. Therefore, the conflicting results on the prognostic role of synchronous disease may be caused by differences among the status of the resection of the primary tumor. The prognostic value of resection of the primary tumor in patients with synchronous metastases is the research question in a new randomized clinical trial of the DCCG.

Due to the negative predictive value of *KRAS* mutations, the use of anti-EGFR antibodies in metastatic CRC is restricted to patients with *KRAS* wild-type tumors. *KRAS* mutation analysis is usually performed on primary tumor tissue because metastatic tissue is often not available. A possible discordance of test results between primary tumor and metastases has been suggested as an explanation for the failure rate of anti-EGFR therapy in patients with *KRAS* wild-type tumors. In **chapter 5** we describe the concordance of the *KRAS* mutation status in 305 primary colorectal tumors and their corresponding liver metastases. *KRAS* mutations were detected in 35.4% of the primary tumors. In 11 cases (3.6%) we observed a discordance between primary tumors and metastases: 5 primary tumors had a *KRAS* mutation with wild-type metastases, 1 primary tumor was wild-type with a *KRAS* mutation in the metastasis, and in 5 cases the primary tumor and the metastases had a different type of *KRAS* mutation. In this largest and most homogenous study to date, we observed a high concordance of *KRAS* mutation status of 96.4% (95% CI 93.6-98.2%) between primary colorectal tumors and their corresponding liver metastases. In only 6 patients (2.0%; 95% CI 0.7-4.2%) the discordance in *KRAS* mutation status was clinically relevant. We concluded that both primary tumors and liver metastases can be used for *KRAS* mutation analysis.

In **chapter 6** we present the results of a sub-analysis where we correlated *KRAS* modification by gene copy number aberrations and miRNAs to clinical outcome in metastatic CRC patients treated with cetuximab containing first-line treatment. In this pilot study, 34 metastatic CRC patients were selected based upon their good (n=17) and poor (n=17) PFS upon treatment with cetuximab in combination with capecitabine, oxaliplatin, and bevacizumab. Good response was associated with 12p12.1 copy number loss, even in patients with a *KRAS* mutation, while copy number gain in wild-type *KRAS* patients was correlated with a poor response. In *KRAS* mutated tumors increased miR-200b and decreased miR-143 expression were associated with a good response. In wild-type *KRAS* patients, miRNA expression did not predict response in a multivariate model. Thus, assessment of *KRAS* copy number aberrations and miRNAs targeting *KRAS* might further optimize the selection of patients eligible for anti-EGFR therapy. However, given the small number of patients included in our study, confirmation in larger series is warranted.

Metastatic disease is the major cause of death in CRC patients. The metastatic process comprises multiple sequential steps, including tumor cell invasion of the microenvironment, entering and survival in the bloodstream, extravasation, proliferation, induction of angiogenesis, and evading apoptotic death at the secondary site. The role of epigenetic regulation in the metastatic pathway remains obscure, therefore we reviewed in **chapter 7** the relationship between miRNAs and the CRC metastatic pathway. We have summarized several miRNAs and their known target genes that are associated with specific steps in the metastatic process,

mainly investigated using '*in vitro*' and '*in vivo*' models. Many of these miRNAs are known to be dysregulated in CRC, but only limited data are available that link miRNA expression to the metastatic human CRC phenotype. To gain better insight in the miRNAs involved in human CRC metastasis, we suggest to compare miRNA expression profiles in primary tumors and metastatic tissue. In addition, to identify miRNAs involved in metastases it is also relevant to compare miRNA expression in primary tumors of patients with and without metastatic disease. The next step is to assess the biological consequences of these miRNAs on genes responsible for metastases, using *in vivo* human CRC models. This approach appears useful and should designate candidate miRNAs for further use as therapeutical targets in CRC treatment.

In addition to the epigenetic alterations reviewed in the previous chapter, also chromosomal aberrations are involved in the metastatic process. It is still a matter of debate how, where, and when the tumor cells acquire the genetic alterations that drive the metastatic process. In **chapter 8** we present the differences of 62 primary colorectal cancers, 62 matched normal specimens, and 68 matched metastases (from liver, lung, ovary, omental, and distant lymph nodes) by high resolution array CGH for DNA copy number changes. Overall patterns of DNA copy number aberrations were highly similar between primary tumors and their metastases, confirming clonality. Additional copy number aberrations in metastases are rare and rather than recurrent they were sporadic for individual patients. The only recurrent differences between primary tumors and their metastases were observed in two chromosomal regions, 6q21 and 8q24.21 encompassing the *MYC* oncogene, that coamplified in three metastases of two patients (3.2%). The co-amplification was confirmed by fluorescent in situ hybridization analysis and did not result from translocation, since no co-localization of the amplified chromosomal regions was observed. From this large array CGH study we showed highly similar patterns of DNA copy number aberrations between primary CRC and their metastases. These observations are consistent with the hypothesis that the metastatic potential is predestined early in the development of the primary tumor.

In **chapter 9** we describe the clinicopathological features and genome-wide chromosomal aberrations associated with organ-specific metastases in CRC. Clinicopathological features were investigated in metastatic CRC patients with exclusively hepatic (n=182) versus extrahepatic (n=139) metastases, who participated in the phase III CAIRO study. A total of 139 primary tumors of patients with hepatic (n=85) and extrahepatic metastases (n=54) were screened for chromosomal aberrations by array CGH, and the findings were validated in an independent set of 80 primary tumors. Moreover, a publicly available database was used to correlate chromosomal aberrations with gene expression, and protein expression was evaluated by immunohistochemistry. Patients with hepatic metastases were significantly more often male, had more frequently an abnormal LDH, primary tumor localization in the colon,

synchronous onset of metastases, T3 tumors, and were less frequently of mucinous histology. No significant difference in clinical outcome was observed between patients with hepatic and extrahepatic metastases. Gain of 20p11 was more frequently observed in patients with hepatic metastases, which was confirmed in an independent dataset. Twelve genes mapping at 20p11 were significantly overexpressed as a consequence of 20p11 copy number gain. *C20orf3* showed the strongest correlation between RNA expression and DNA copy number. This was reflected in a significantly higher protein expression present in patients with hepatic metastases (59%; n=325) versus extrahepatic metastases (41%; n=256) ($p=0.01$). The possible role of this hepatic metastasis-associated gene in specific steps of the hepatic metastatic process needs to be functionally validated. This could result in the development of new prognostic markers that could help identifying patients who are most likely to develop liver metastases and the development of liver-specific anti-metastatic therapies in the future.

GENERAL CONCLUSION

This thesis focuses on translational aspects of CRC metastases. CRC is a heterogeneous disease in many ways, as is reflected by the large number of already known prognostic factors. We investigated several other factors with potential prognostic value in metastatic CRC, and observed that mucinous histology of the primary tumor is an important negative prognostic marker for clinical outcome. Synchronous presentation of metastases at diagnosis is considered to be of worse prognostic value compared with metachronous metastases. We have challenged previous data by performing a more homogeneous study and concluded that the onset of metastases is not of prognostic value in metastatic CRC. We also established the relationship between lymph node retrieval and outcome in rectal cancer patients. The use of these and other established prognostic markers in clinical practice may individualize patient care. In addition, optimization of predictive markers is necessary to initiate an individualized treatment. The identification of a *KRAS* mutation as a predictive marker for response to anti-EGFR agents has been one of the most important achievements in CRC research in recent years. The high concordance rate in *KRAS* mutation status that we established between primary tumor and metastatic tissue justifies testing in the primary tumor before initiating anti-EGFR therapy. We excluded a discordance in test results as explanation for the failure rate of anti-EGFR therapy in patients with *KRAS* wild-type tumors. Other regulatory mechanisms of *KRAS*, such as miRNAs and copy number aberration, are probably important in predicting response to anti-EGFR agents and could further improve the selection of patients for anti-EGFR therapy. However, our results are only hypothesis-generating and should be further tested in larger patient cohorts. To develop new targeted therapies in CRC it is essential to identify (epi)genetic alterations responsible for metastatic progression. Therefore we reviewed miRNA profiles and performed array CGH profiling, resulting in a better comprehension of the metastatic behavior. In the near future, these basic research findings should be translated to the development of new targeted treatments which can be used in the oncology clinic. In addition, the role of organ-specificity in the pathway of CRC metastases is even more complex and still largely unknown. We identified an array CGH profile including the protein encoding gene *C20orf3* that is overrepresented in primary CRC with a preferential metastatic pattern to the liver. This finding may contribute to the development of new prognostic markers that identify patients who are most likely to develop liver metastases and to the development of liver-directed cancer therapies in the future.



Chapter 10

Nederlandse samenvatting

SAMENVATTING

Dikke- en endeldarmkanker (colorectaal carcinoom) is één van de meest voorkomende vormen van kanker in de westerse wereld en de incidentie ervan neemt toe. De meeste colorectaal carcinomen ontstaan als goedaardige afwijkingen die door een opeenvolging van genetische defecten kwaadaardig worden. Deze kwaadaardige tumorcellen kunnen van de oorspronkelijke darmtumor losraken en via de bloedvaten of de lymfevaten terecht komen in andere organen. Dit proces wordt uitzaaiing of metastasering genoemd. De moleculaire mechanismen die verantwoordelijk zijn voor metastasering zijn nog grotendeels onbekend. Meer kennis over de biologie van metastasering zal ons in staat kunnen stellen om meer effectieve behandelingen te ontwikkelen. Ongeveer 50% van alle patiënten met een colorectaal carcinoom ontwikkelt metastasen, met name in de lever en longen. Een beperkt deel van patiënten met levermetastasen komt in aanmerking voor chirurgische resectie van deze metastasen, waarbij genezing in minder dan de helft van deze patiënten mogelijk is. Bij het grootste deel van patiënten met metastasen is genezing dus niet mogelijk, maar kan er wel een aanzienlijke overlevingswinst behaald worden met medicamenteuze therapie. Deze behandeling bestaat uit een combinatie van chemotherapie (fluoropyrimidines, oxaliplatin en irinotecan) en zogenaamde targeted therapie (VEGF en EGFR antilichamen). Laatstgenoemde middelen remmen specifieke groeisignalen in de kankercel waardoor processen zoals proliferatie (vermenigvuldiging), angiogenese (bloedvat nieuwvorming), en het uitblijven van apoptose (geprogrammeerde celdood) geremd kunnen worden. De medicamenteuze behandeling van het gemetastaseerde colorectaal carcinoom kent een enorme variatie in effectiviteit op de ingestelde therapie. Deze variatie wordt bepaald doordat er tussen patiënten verschillen zijn in prognostische en predictieve factoren. Prognostische factoren geven informatie over het ziektebeloop onafhankelijk van de behandeling, en predictieve factoren voorspellen hoe een tumor op een bepaalde behandeling reageert. Het vergroten van de kennis op het gebied van deze factoren kan de behandeling van een kankerpatiënt individualiseren en optimaliseren.

In **hoofdstuk 2** beschrijven we de prognostische waarde van het aantal verkregen lymfeklieren in 1227 patiënten met endeldarmkanker (rectumcarcinoom) die behandeld zijn in een gerandomiseerde fase III studie. In deze studie zijn patiënten met een rectumcarcinoom gerandomiseerd voor TME chirurgie met of zonder een kortdurende preoperatieve bestraling. Volgens de huidige standaard dienen tenminste 10 regionale lymfeklieren verkregen te worden om een betrouwbare inschatting van de prognose te kunnen maken. Het mediane aantal onderzochte regionale lymfeklieren in alle patiënten die deelnamen aan deze studie is 7. Bij patiënten die behandeld zijn met preoperatieve bestraling worden minder lymfeklieren verkregen in vergelijking met de patiënten die niet bestraald zijn voor de operatie. Er hebben 49 pathologie

laboratoria deelgenomen aan deze studie en verrassend genoeg is het aantal verkregen lymfeklieren zeer verschillend tussen pathologen en tussen laboratoria. De volgende patiënt- en tumorgerelateerde factoren zijn geassocieerd met een te laag aantal verkregen lymfeklieren: leeftijd ouder dan 60 jaar, overgewicht, kleine tumor diameter, lager T stadium, slechte differentiatie, afwezigheid van lymfekliermetastasen, en weinig tot geen ontstekingsreactie rondom de primaire tumor. Patiënten met lymfeklieren waarin zich geen uitzaaiingen bevinden hebben een langer ziektevrij interval als er minimaal 8 lymfeklieren verkregen waren, in tegenstelling tot patiënten waarbij 7 of minder lymfeklieren verkregen zijn. Geconcludeerd kan worden dat het aantal onderzochte lymfeklieren afhankelijk is van meerdere factoren die gerelateerd zijn aan de patiënt, tumor, en behandeling. Het streven naar een goede opbrengst (minimaal 8 lymfeklieren) is noodzakelijk voor adequate stadiëring daar dit de prognose van een rectumcarcinoom patiënt verbetert.

Mucineuze adenocarcinomen zijn een histologisch subtype van het colorectaal carcinoom, waarvan de incidentie 5-15% bedraagt. De tumorcellen van een mucineus adenocarcinoom produceren extracellulair slijm, wat meer dan 50% van het tumor volume omvat. Het adenocarcinoom is een histologisch subtype dat nauwelijks slijm produceert en vormt de meerderheid binnen het colorectaal carcinoom (90%). In **hoofdstuk 3** worden de verschillen besproken tussen patiënten met een gemetastaseerd mucineus adenocarcinoom en een gemetastaseerd adenocarcinoom. Hierbij is gekeken naar klinische karakteristieken, pathologische kenmerken van de primaire tumor, en uitkomst van behandeling. De patiënten zijn geselecteerd uit twee grote gerandomiseerde fase III studies, en betreft de grootste studie op dit gebied tot nu toe. Patiënten met een mucineus adenocarcinoom (n=99) zijn ouder, hebben vaker een normaal serum LDH, extrahepatische lokalisatie van metastasen, een grote diameter van de primaire tumor, en een hoger T stadium vergeleken met patiënten met een adenocarcinoom (n=911). *BRAF* mutaties en afwijkingen in het DNA herstel mechanisme worden vaker geobserveerd in patiënten met een mucineus adenocarcinoom. Totale overleving, progressie vrije overleving en respons percentages zijn slechter in patiënten met een mucineus adenocarcinoom die behandeld worden met chemotherapie en targeted therapie in vergelijking met de adenocarcinoom groep. In de multivariate analyses voor de totale overleving is mucineuze histologie een sterke onafhankelijke negatief prognostische marker bij patiënten met een gemetastaseerd colorectaal carcinoom. Op grond van onze resultaten stellen wij voor om mucineuze histologie te gebruiken als een stratificatieparameter bij klinische studies met colorectaal carcinoompatiënten.

Op het moment van de initiële diagnose heeft 20% van de patiënten met een colorectaal carcinoom reeds synchrone metastasen. In totaal ontwikkelt circa 50% van de patiënten metachrone metastasen, welke meestal binnen drie jaar na het stellen van de diagnose

optreden. In de literatuur wordt het hebben van synchrone metastasen geassocieerd met een slechtere prognose in vergelijking met patiënten met metachrone metastasen. In **hoofdstuk 4** wordt een analyse beschreven waarbij de klinische parameters, pathologische kenmerken van de primaire tumor, en overleving vergeleken worden tussen patiënten met synchrone versus metachrone colorectale metastasen. Vergeleken met de groep met metachrone metastasen (n=270), hebben de patiënten met synchrone metastasen (n=280) vaker een jongere leeftijd, een verhoogd serum LDH, de lever als het meest betrokken orgaan van metastasering, de primaire tumor in het colon, een slechte performance status, een grote diameter van de primaire tumor, een hoger T en N stadium, weinig tot geen lymfoïde reactie, en een diffuus infiltratiepatroon. Van de parameters die significant vaker voorkomen bij patiënten met synchrone metastasen zijn een verhoogd serum LDH, slechte WHO performance status, primaire tumorlokalisatie in het colon, en het T stadium geassocieerd met een slechte overleving in de totale studiepopulatie. Echter, ondanks het significant vaker voorkomen van factoren geassocieerd met een slechte prognose in de groep met synchrone metastasen, is er geen verschil in overleving tussen patiënten met synchrone en metachrone metastasen waarbij de primaire tumor verwijderd is. Een mogelijke verklaring hiervoor is dat patiënten met metachrone metastasen frequenter adjuvante chemotherapie hebben gehad in vergelijking met die met synchrone metastasen, wat theoretisch zou kunnen leiden tot chemoresistentie. Echter, vele studies waaronder de CAIRO studie laten geen onafhankelijk prognostische waarde zien voor eerder gegeven adjuvante chemotherapie.

In eerdere studies is de uitkomst van patiënten met synchrone metastasen wel vaak slechter ten opzichte van de groep met metachrone metastasen. In vergelijking met deze studies laat onze analyse één belangrijk verschil zien, namelijk dat wij alleen patiënten met synchrone metastasen hebben geanalyseerd die eerder een resectie van de primaire tumor hebben ondergaan. Dezelfde analyse in de totale CAIRO studiepopulatie, dus ook de patiënten met synchrone metastasen die geen resectie van de primaire tumor hebben ondergaan, toont wel een overlevingswinst voor patiënten met metachrone metastasen. In een dergelijke analyse is er echter een potentiële bias van de resectie van de primaire tumor aanwezig, daar alle patiënten met metachrone metastasen deze resectie ondergingen, hetgeen niet het geval is bij de synchrone metastasen. Onze analyse is de eerste studie die bij patiënten met een verwijderde primaire tumor aantoont dat de overleving bij synchrone versus metachrone metastasen niet verschillend is als gestart wordt met een medicamenteuze behandeling. De prognostische waarde van een resectie van de primaire tumor bij patiënten met synchrone metastasen is thans onderwerp van prospectief onderzoek.

Het is gebleken dat patiënten met een *KRAS* mutatie in het tumorweefsel niet responderen op behandeling met EGFR remmers, zoals cetuximab en panitumumab. Het is daarom alleen zinvol om patiënten met een *KRAS* wild type tumor te behandelen met anti-EGFR therapie.

KRAS mutatie analyse wordt meestal uitgevoerd op weefsel van de primaire tumor, omdat weefsel van de metastasen meestal niet beschikbaar is. Of het uitmaakt in welk type tumorweefsel de test wordt uitgevoerd is tot op heden niet duidelijk vanwege de heterogene studies in kleine patiënten populaties. In **hoofdstuk 5** beschrijven we in 305 primaire colorectale tumoren en corresponderende levermetastasen de overeenkomst in *KRAS* mutatie status. Het *KRAS* oncogen is gemuteerd in 35.4% van de primaire tumoren. In 11 patiënten wordt een discordantie aangetoond tussen de primaire tumor en de metastase; 5 patiënten met een *KRAS* mutatie in de primaire tumor en een wild type in de metastase, 1 patiënt met een *KRAS* wild type in de primaire tumor en een mutatie in de metastase, en 5 patiënten met een verschillende *KRAS* mutatie tussen de primaire tumor en metastase. Concluderend is er sprake van een hoge concordantie (96.4%) in *KRAS* mutatie status tussen de primaire tumor en de levermetastase. Het klinische relevante percentage ligt nog hoger als de 5 patiënten met een verschillende *KRAS* mutatie niet meegeteld worden, namelijk 98%. Uit deze tot nu toe grootste studie concluderen we dat zowel weefsel van de primaire tumor als van de metastase gebruikt mag worden voor de *KRAS* mutatie analyse. Daarnaast stellen we vast dat het niet responderen op behandeling met EGFR remmers bij patiënten met een *KRAS* wild type tumor niet kan worden verklaard door discordantie in de *KRAS* mutatie status van de primaire tumor en het doelwit van de anti-EGFR behandeling, de metastasen.

Een andere verklaring voor de afwezige respons op anti-EGFR therapie in *KRAS* wild type patiënten ligt op het terrein van de regulatie van *KRAS*. Naast de puntmutatie kunnen ook andere regulatie mechanismen de expressie van *KRAS* beïnvloeden, waarbij veranderingen in het aantal *KRAS* kopieën en microRNAs (miRNAs) die aangrijpen op *KRAS* onze speciale interesse hebben. MiRNAs zijn kleine stukjes RNA die niet coderen voor een eiwit, maar wel de expressie van andere genen kunnen beïnvloeden. In **hoofdstuk 6** correleren we afwijkingen in het aantal *KRAS* kopieën en miRNAs die aangrijpen op *KRAS*, met de klinische uitkomst van patiënten die behandeld zijn met cetuximab bevattende eerstelijns behandeling. In deze studie hebben we 34 patiënten met een gemetastaseerd colorectaal carcinoom geselecteerd op basis van een lange (n=17) en korte (n=17) progressie vrije overleving na behandeling met cetuximab, bevacizumab en chemotherapie. Bij deze patiënten hebben we gebruik gemaakt van array comparative genomic hybridization (array CGH) om het aantal *KRAS* kopieën vast te stellen en kwantitatieve RT-PCR om de miRNA expressie te bepalen. Een lange progressievrije overleving is geassocieerd met het verlies van kopieën van het *KRAS* gen, zelfs in patiënten met een *KRAS* mutatie. Daarnaast gaat een toename van het aantal *KRAS* kopieën gepaard met een korte progressievrije overleving in patiënten met een *KRAS* wild type. Verhoogde expressie van miRNA-200b en verlaagde expressie van miRNA-143 voorspellen een lange progressievrije overleving op cetuximab bevattende behandeling bij patiënten met een *KRAS* mutatie. In een multivariate analyse lijkt miRNA expressie geen effect te hebben op de cetuximab respons in

patiënten met een *KRAS* wild type tumor. Wij concluderen dat het vaststellen van chromosomale afwijkingen in *KRAS* en miRNAs die aangrijpen op *KRAS* de selectie van patiënten voor anti-EGFR therapie verder kan optimaliseren. Gezien de beperkte aantallen patiënten en het feit dat de patiënten naast cetuximab ook behandeld zijn met chemotherapie en bevacizumab dienen deze resultaten bevestigd te worden in toekomstige studies.

Het ontwikkelen van metastasen op afstand is de belangrijkste oorzaak van kanker-gerelateerde sterfte bij patiënten met een colorectaal carcinoom. Metastasering is een gefaseerd proces bestaande uit invasie, proliferatie, extravasatie, en angiogenese. MiRNAs zijn betrokken bij het ontstaan van kanker, maar de rol in het proces van metastasering bij het colorectaal carcinoom is grotendeels onbekend. In **hoofdstuk 7** wordt een overzicht gegeven van de tot nu toe bekende gegevens over de relatie tussen miRNA expressie en alle facetten van de metastase cascade bij patiënten met een colorectaal carcinoom. De miRNAs zijn met name geselecteerd op onderzoek met '*in vitro*' studies en diermodellen. Vervolgens hebben we meer in detail gezocht naar gegevens over (epi)genetische afwijkingen op deze specifieke miRNA loci. We hebben voor deze benadering gekozen omdat er in de huidige literatuur maar weinig gegevens beschikbaar zijn die miRNA expressie direct correleren met het metastatisch fenotype. Om een beter inzicht te krijgen in de rol van miRNA expressie in het metastaseringsproces adviseren wij om het miRNA expressie profiel van primaire tumoren te vergelijken met de corresponderende metastasen op afstand. Daarnaast is het ook zinvol om het miRNA expressie profiel van primaire tumoren te vergelijken bij patiënten met en zonder metastasen op afstand. De aldus geselecteerde miRNAs moeten vervolgens in humane colorectaal carcinoom modellen functioneel getest worden om zodoende het biologische mechanisme te kunnen verklaren. Deze benadering zou er toe moeten leiden dat doelgerichte therapie tegen miRNAs een nieuwe therapiemodaliteit gaat vormen van het colorectaal carcinoom.

Zoals duidelijk is uit het voorafgaande hoofdstuk is metastasering een ingewikkeld proces waarbij de relevante chromosomale gebieden en genen nog niet zijn geïdentificeerd. Om te onderzoeken of er in het proces van metastasering nieuwe chromosomale afwijkingen ontstaan hebben we in **hoofdstuk 8** het aantal en de soort chromosomale afwijkingen in de metastase vergeleken met de bijbehorende primaire colorectale tumor van een patiënt. Hiervoor hebben we 62 patiënten geselecteerd waarbij we de 62 primaire tumoren en de 68 gepaarde metastasen (lever, long, ovarium, omentum, lymfeklier op afstand) geanalyseerd hebben middels array CGH op het vóórkomen van chromosomale afwijkingen. We hebben onze bevindingen gevalideerd met behulp van een bestaande beschikbare array CGH dataset en fluorescentie in situ hybridisatie (FISH). Bij deze laatste techniek kleuren we de chromosoomdelen aan die we vervolgens kunnen bestuderen onder een fluorescentie microscoop. De resultaten van onze studie laten zien dat het array CGH profiel van de metastase en de

gepaarde primaire tumor sterk met elkaar overeenkomen. In de clusteranalyse, clustert de meerderheid (56 paren) naast elkaar wat betekent dat ze meer op elkaar lijken dan op een andere tumor. De tweede bevestiging voor clonaliteit krijgen we door het aantal kopieën van de primaire tumoren af te trekken van het aantal kopieën van de metastase. Dit hebben we voor elk chromosomaal gebied gedaan. Bij de meerderheid van de chromosomale gebieden wijkt het aantal kopieën tussen de metastase en de primaire tumor niet van elkaar af. Echter, bij twee patiënten werd een bijzondere toename van het aantal kopieën (amplificatie) op twee gebieden in de metastase gezien, welke niet aanwezig waren in de primaire tumor. Deze sterke amplificatie omvat het gebied waar ook het *MYC* oncogen ligt die betrokken is bij de pathogenese van het colorectaal carcinoom. Deze bevinding is intrigerend, maar zien we slechts in twee van de 62 patiënten. Uit deze grote array CGH studie concluderen we dat metastasen geen extra chromosomale afwijkingen hebben in vergelijking met de corresponderende primaire tumor. Deze bevinding is zeer relevant voor het begrip van het model van metastasering, waarbij we aannemen dat de genen die verantwoordelijk zijn voor metastasering al vroeg in de primaire tumor aanwezig zijn.

In **hoofdstuk 9** onderzoeken we een aantal klinische, histopathologische, en chromosomale afwijkingen die betrokken zijn bij orgaanspecifieke metastasering van het colorectaal carcinoom. De klinische en pathologische kenmerken worden vergeleken tussen colorectaal carcinoom patiënten met alleen levermetastasen (n=182) versus patiënten met alleen metastasen buiten de lever (extrahepatische metastasen)(n=139). In 139 primaire tumoren van patiënten met alleen levermetastasen (n=85) en patiënten met alleen extrahepatische metastasen (n=54) is het gehele genoom geanalyseerd op chromosomale afwijkingen. Deze bevindingen worden vervolgens gevalideerd in een tweede set van 80 primaire colorectale tumoren. We maken gebruik van eerder verrichte genexpressie studies om genen te selecteren op de belangrijke chromosomale gebieden, en immunohistochemie om het effect op eiwit niveau te evalueren. Patiënten met alleen levermetastasen zijn vaker van het mannelijk geslacht, hebben vaker een verhoogd serum LDH, primaire tumor lokalisatie in het colon, synchrone presentatie van metastasen, T3 tumoren, en zijn minder vaak van mucineuze origine in vergelijking met patiënten met alleen extrahepatische metastasen. Er is geen verschil in progressie vrije overleving en totale overleving tussen patiënten met alleen lever versus alleen extrahepatische metastasen. Na evaluatie van het gehele genoom op chromosomale instabiliteit wordt een toename van het aantal kopieën (gain) van chromosoom 20p11 vaker geobserveerd in patiënten met levermetastasen versus patiënten met extrahepatische metastasen, hetgeen wij hebben bevestigd in een onafhankelijke dataset. Genexpressie profilering toont 12 genen op chromosoom 20p11 aan, die een significante overexpressie hebben in patiënten met 20p11 gain versus patiënten zonder 20p11 gain. Van deze 12 genen, heeft *C20orf3* de sterkste correlatie tussen RNA expressie en de mate van chromosomale instabiliteit. Bij patiënten met

levermetastasen (n=325) zien we ook daadwerkelijk vaker aanwezigheid van C20orf3 eiwitexpressie in vergelijking met patiënten met extrahepatische metastasen (n=256). Het biologische mechanisme achter 20p11 gain en C20orf3 expressie in orgaanspecifieke metastasering is nog onbekend. Deze marker zou van prognostische waarde kunnen zijn en is mogelijk een nieuw aangrijpingspunt bij de preventie of behandeling van levermetastasen bij patiënten met een colorectaal carcinoom.

ALGEMENE CONCLUSIE

Dit proefschrift richt zich op de translationele aspecten van metastasering bij patiënten met een colorectaal carcinoom. Het gemetastaseerd colorectaal carcinoom manifesteert zich als een heterogene ziekte, met name als we kijken naar prognose. Wij hebben enkele factoren onderzocht die dit verschil in prognose zouden kunnen bepalen. Mucineuze histologie van de primaire tumor is een belangrijke negatieve voorspeller voor de overleving van een patiënt met een gemetastaseerd colorectaal carcinoom. Uit eerdere studies wordt het hebben van synchrone versus metachrone metastasen als prognostisch slecht ervaren. Wij hebben deze bevinding weerlegd door in een homogene groep van colorectaal carcinoom patiënten aan te tonen dat er geen verschil is in de overleving tussen patiënten met synchrone versus metachrone metastasen. Een andere prognostische factor is het aantal lymfeklieren dat verkregen wordt uit een resectie preparaat. Het verkrijgen van lymfeklieren is afhankelijk van meerdere patiënt-, tumor-, en behandelingsgerelateerde factoren. Echter, een goede opbrengst (minimaal 8 lymfeklieren) is noodzakelijk voor adequate stadiëring hetgeen van belang is om de prognose in te kunnen schatten van een rectumcarcinoom patiënt. Verder onderzoek moet leiden tot de identificatie van de meest relevante klinische en pathologische prognostische factoren. Deze factoren kunnen aangevuld worden met (epi)genetische prognostische markers waardoor een prognostisch model opgesteld kan worden. Met een dergelijk model lijkt het mogelijk om een betere inschatting te maken van de overleving per individuele patiënt, hetgeen belangrijke consequenties heeft voor de algemene praktijk.

Het inschatten van de overleving met behulp van prognostische markers maakt individualisering van de behandeling mogelijk, echter het ontdekken van predictieve markers maakt een individueel aangepaste behandeling werkelijkheid. Het aantonen van een mutatie in het *KRAS* oncogen als negatieve predictieve marker voor anti-EGFR therapie is een grote doorbraak van de afgelopen jaren. Het is tot op heden onduidelijk of de *KRAS* mutatie status in weefsel van zowel de primaire tumor als de (veel minder vaak beschikbare) metastase vastgesteld moet worden voorafgaand aan de start van anti-EGFR therapie. Gezien de hoge concordantie in

KRAS mutatie status tussen de primaire tumor en de metastase, is het valide om alleen de primaire tumor te testen. Daarnaast geven deze resultaten aan dat een verschil in de uitslag van de *KRAS* mutatietest tussen de primaire tumor en de metastase dus geen verklaring is voor het feit dat een aanzienlijk aantal *KRAS* wild type patiënten toch niet reageren op anti-EGFR therapie. Ook andere potentiële predictieve markers, zoals PTEN expressie, *PIK3CA* mutaties en kiembaan veranderingen, kunnen de afwezige respons in *KRAS* wild type patiënten niet volledig verklaren. Veel centra in de wereld zijn op dit moment bezig om nieuwe predictieve markers voor anti-EGFR therapie op te sporen. Echter, naast het zoeken naar nieuwe markers verdient de predictieve waarde van *KRAS* verdere optimalisatie. Een puntmutatie in het *KRAS* oncogen is niet de enige regulator van de *KRAS* activiteit, want uit *in vitro* studies blijken andere (epi)genetische afwijkingen ook de expressie te reguleren. Uit onze hypothese genererende studie blijkt dat het bepalen van het aantal *KRAS* kopieën en de expressie van miRNAs die aangrijpen op *KRAS*, de selectie van patiënten voor anti-EGFR therapie kan verbeteren. Onze uitkomsten moeten in ander onderzoek gevalideerd worden om de werkelijke waarde van het aantal *KRAS* kopieën en miRNAs op *KRAS* te evalueren voordat dit toegepast kan worden in de klinische praktijk.

Het proces van metastasering is zeer complex, maar het is essentieel om meer inzicht te krijgen in specifieke chromosomale afwijkingen en genen die betrokken zijn bij de metastasering van het colorectaal carcinoom. Dit inzicht wordt verkregen door in de primaire tumor en de gepaarde metastase te kijken naar chromosomale afwijkingen en veranderingen in de miRNA expressie. Op deze manier komen (epi)genetische afwijkingen aan het licht, die mogelijk de drijvende kracht zijn achter het ontstaan van metastasen op afstand. Nog een stap verder en complexer is het proces van orgaanspecifieke metastasering. Orgaanspecifieke metastasering berust onder meer op de mechanische theorie waarbij de veneuze bloedafvoer van de primaire tumor het orgaan van metastase bepaald. Ons onderzoek laat echter zien dat deze theorie niet een sluitende verklaring is maar dat orgaanspecifieke metastasering in ieder geval mede bepaald wordt door het chromosomaal profiel van de primaire tumor. We hebben een array CGH profiel geïdentificeerd welke geassocieerd lijkt te zijn met primaire tumoren die naar de lever metastaseren in vergelijking met primaire tumoren die niet naar de lever metastaseren. Deze gegevens zijn om meerdere redenen zeer belangrijk. Ten eerste kan het bepalen van dit profiel in niet-gemetastaseerde colorectale tumoren de follow-up individualiseren. Daarnaast biedt het inzicht in en reden tot verder onderzoek om het biologische mechanisme van orgaanspecifieke metastasering te ontrafelen. Dit onderzoek zal moeten bestaan uit functionele studies, waarbij met behulp van *in vitro* en *in vivo* modellen nieuwe targets worden ontwikkeld als wapen in de strijd tegen het gemetastaseerd colorectaal carcinoom.



Chapter 10

Publication list

PUBLICATION LIST

- M. Koopman, G.A. Kortman, **L.J.M. Mekenkamp**, M.J. Ligtenberg, N. Hoogerbrugge, A.F. Antonini, C.J.A. Punt, J.H.J.M. van Krieken. Deficient mismatch repair system in patients with sporadic advanced colorectal cancer. *Br J Cancer* 2009;100:266-273.
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Chapter 10

Dankwoord

DANKWOORD

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Chapter 10

Curriculum vitae

CURRICULUM VITAE

Leonie Mekenkamp werd op 12 februari 1982 geboren te Hertme (Overijssel). In 2000 behaalde zij haar VWO diploma aan het Twickel College te Hengelo (Overijssel). Vanaf 2000 studeerde zij Biomedische Wetenschappen aan de Katholieke Universiteit Nijmegen (thans Radboud Universiteit Nijmegen). Na het behalen van de Bachelor of Science is zij in 2003 gestart met de studie Geneeskunde. Tijdens deze studie heeft zij als student-assistent werkzaamheden verricht op de afdeling pathologie, o.a. ten behoeve van de CAIRO studies van de Dutch Colorectal Cancer Group (DCCG).

Na het behalen van het artsexamen in 2008 begon zij als arts (AGNIO) bij de afdeling Interne Geneeskunde van het Medisch Spectrum Twente te Enschede, alwaar zij datzelfde jaar de opleiding tot internist startte (opleiders prof. dr. R.O.B. Gans; dr. W. Smit). In november 2009 werd zij door de DCCG aangenomen als onderzoeker, en werkte zij op de afdeling Pathologie en Medische Oncologie in het UMC St Radboud aan het onderzoek dat in dit proefschrift is beschreven. Zij deed dit onder begeleiding van prof. dr. C.J.A. Punt (afdeling Medische Oncologie), prof. dr. I.D. Nagtegaal (afdeling Pathologie) en dr. M. Koopman (afdeling Medische Oncologie en later UMC Utrecht). In november 2011 heeft zij de opleiding tot internist hervat en daarna zal zij de opleiding tot medisch oncoloog starten in het AMC te Amsterdam (opleider dr. A.M. Westermann).